Adrenergic and Cholinergic Stimulation of Cortisol Production in Primary Cultures of Bovine Adrenocortical Zona Fasciculata / Reticularis Cells

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The following thesis contains the results of my own research, and was written and composed by myself.

In memory of Janet, who was always an inspiration.

"God has also set eternity in the hearts of men, yet they cannot fathom what he has done from beginning to end."

(Ecclesiastes 3v11)

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Adrenergic and Cholinergic Stimulation of Cortisol Production in Primary Cultures of Bovine Adrenocortical Zona Fasciculata / Reticularis Cells

In addition to the classical hormonal regulators of adrenocortical steroidogenesis, adrenocorticotrophic hormone (ACTH) and angiotensin II (AII), there is evidence that the adrenal cortex may also be influenced by adrenergic and cholinergic control.

Initial characterisation of primary cultures of bovine adrenocortical zona fasciculata / reticularis cells, prepared by a collagenase digestion procedure, showed that $ACTH_{1-24}$ and AII stimulated cortisol production from both freshly isolated (day 1) and cultured (day 2 - day 5) cells. Peak cortisol production was seen between days 3 and 4 for these agonists. Morphological analysis, by light and electron microscopy, of day 1 and 3 cells, showed that day 3 cells had improved integrity of ultrastructure and increased lipid deposits compared to day 1 cells.

Adrenergic agonists stimulated cortisol production in a dose-dependent manner from cultured cells, but failed to stimulate cortisol production from freshly isolated cells. Cholinergic agonists stimulated cortisol production in a dose-dependent manner from both freshly isolated and cultured cells. Adrenergic and cholinergic agonists also showed peak cortisol production between days 3 and 4.

Using specific alpha and beta-adrenergic agonists and antagonists, it was shown that adrenergic stimulation of cortisol production from cultured cells was mediated by beta-adrenergic receptors. Schild analysis, using the specific $beta_1$-adrenergic antagonist, practolol, and the specific $beta_2$-adrenergic antagonist, ICI118,551, identified these receptors as $beta_1$-

adrenoceptors. Adrenergic agonists were shown to stimulate steroidogenesis in cultured cells via a cyclic AMP dependent mechanism. These agonists had no effect on turnover of cellular phosphoinositides. Additionally, the adrenergic response of these cells exhibited homologous desensitisation.

Using specific cholinergic agonists and antagonists, it was shown that M_3-cholinergic receptors were responsible for mediating stimulation of cortisol production from cultured cells produced by cholinergic agonists. It was also shown that cholinergic agonists stimulated steroidogenesis in cultured cells via activation of phospholipase C, and that these agonists had no effect on cellular cyclic AMP levels.

Abbreviations

ACh	Acetylcholine
ACTH	Adrenocorticotrophic hormone
Adr	Adrenaline
AII	Angiotensin II
B	Basal
BSA	Bovine serum albumin
CCh	Carbamyl choline (carbachol)
CPSR (1-5)	Controlled process serum replacement
Cyclic AMP	Adenosine 3',5'-cyclic mono-phosphate
CRH	Corticotropin releasing hormone
CNS	Central nervous system
DHA	Dihydro alprenolol
DNA	Deoxy ribo nucleic acid
DNAase	Deoxy ribo nucleotidase
EBS	Earle's balanced salt solution
EDTA	Ethylene diamine tetra acetic acid
FCS	Fetal calf serum
HCS	Heat treated fetal calf serum
HEPES	N-2-hydroxyethyl piperazine N'-2-ethane sulfonic acid
HPLC	High pressure liquid chromatography
Isop	Isoprenaline
IU/ml	International units per millilitre
NAdr	Noradrenaline
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
mAChR	Muscarinic acetylcholine receptor
mRNA	Messenger ribo-nucleic acid
nAChR	Nicotinic acetylcholine receptor
QNB	Quinuclidyl benzylate
SCP	Sterol carrier protein
TRIS	2-amino 2(hydroxymethyl)propane 1,3 diol
ZF	Zona fasciculata
ZFR	Zona fasciculata / reticularis
ZG	Zona glomerulosa
ZR	Zona reticularis
v/v	volume per volume
w/v	weight per volume
%CV	% Coefficient of Variation

Standard chemical symbol and volume / weight abbreviations used throughout

1 Introduction

1.1 Introduction

The work in this thesis is based on a study of steroidogenesis (primarily cortisol production) in bovine adrenal zona fasciculata / reticularis (ZFR) cells both after initial collagenase isolation and during primary culture, concentrating particularly on mechanisms of adrenergic and cholinergic control.

Many reviews exist already on steroidogenesis in the adrenal cortex of different species, and its regulation by the classical secretagogues: adrenocorticotrophic hormone (ACTH); angiotensin II (AII); potassium ions (K^+) and serotonin. Comparatively little is known, however, about the effects of adrenergic and cholinergic agonists on steroidogenesis in the adrenal cortex.

By way of an introduction, a general description of the adrenal gland - structure and function - will be given, followed by a more detailed review of the current knowledge of the control of cortisol production in the zona fasciculata / reticularis. As the main concerns of this thesis are mechanisms of adrenergic and cholinergic control of adrenal steroidogenesis, an introduction will be given to adrenergic and cholinergic mechanisms in general, the literature on adrenergic and cholinergic mechanisms in the adrenal cortex will be reviewed and the physiological relevance of these mechanisms will be discussed.

In addition to a detailed description of the setting-up and characterisation of a primary culture system for bovine adrenocortical zona fasciculata / reticularis cells, this thesis aims to address the following two

questions:-

(1) What molecular mechanisms are involved in adrenergic and cholinergic stimulated cortisol production in the zona fasciculata / reticularis, and how do these relate to the mode of action of other adrenocortical secretagogues?

(2) What relevance do adrenergic and cholinergic stimulation of cortisol production have to the overall picture of control of cortisol production in the adrenal zona fasciculata / reticularis?

1.2 The Adrenal Cortex - General background

1.2.1 Anatomy

The adrenal glands (*ad*: to, *renes*: kidney. Latin) lie in close proximity to the kidney in mammals. In humans, they are found in the retroperitoneum: the left gland at the tail of the spleen, and the right gland close to the inferior vena cava in the supra-renal berth (Silverman & Lee, 1989). The gland consists of two morphologically and functionally distinct regions, the outer steroid-synthesising cortex, and the inner catecholamine-producing medulla. In most species there is a clear distinction between these regions, though in the human the cortex and the medulla are convoluted (Neville & O'Hare, 1982, Ch 4). Though the medulla and cortex form a single body in mature terrestrial mammals, in the early stages of embryonic growth they are separate, the cortex of mesodermal origin, and the medulla of ectodermal origin (Weinkove & Anderson, 1985, Ch 10)

1.2.2 Interactions Between the Adrenal Cortex and Medulla

In the human The adrenal receives its blood supply from direct branches of the aorta, and from the inferior phrenic and renal arteries. Blood flows inwards, draining from a central vein, to the ipsilateral renal vein on the right hand side, and to the inferior vena cava on the left hand side (Silverman & Lee, 1989). In rat and bovine It is believed that the medulla receives its blood supply almost exclusively from the cortex (Vinson *et al*, 1985), and, indeed, corticosteroids are known to have effects on adrenaline synthesis and release in the medulla (Ungar & Phillips, 1983).*

It is possible that the cortex and medulla are linked in other ways. For example, Carballeira & Venning (1964) have suggested that the medulla has the ability to complete glucocorticoid biosynthesis and Soliman & Kolta (1981) have shown that, though adrenaline apparently had no effect on intact adrenal glands *in vitro*, it did stimulate steroidogenesis in regenerating medulla-ectomised rat adrenal gland. Also, the medulla and cortex are both involved in the mammalian stress response: the medulla producing adrenaline in the "flight or fight" response, and the cortex releasing cortisol classically in response to raised ACTH levels after, for example, surgery (Weinkove & Anderson, 1985, Ch 10). Although both cortex and medulla may display this concerted reaction to stress, these responses are not necessarily linked. Goldstein *et al* (1982) have shown that the stress induced by dental extraction produces a rise in plasma catecholamine concentration, but no significant rise in plasma cortisol levels.

1.2.3 Zonation of the Adrenal Cortex

The human adrenal gland was described as early as 1611 (Bartholinus), although little was then known of its function. In 1856,

* In the human, in the region of the adrenal cuff, the zona glomerulosa is next to the adrenal vien and blood flow is likely to be from medulla to cortex.

Brown-Sequard demonstrated that the adrenal was essential to life, and Wheeler and Vincent showed in 1917 that the ability to sustain life was due to the outer cortex and not the inner medulla. The now accepted division of the cortex into the three zones, namely, zona glomerulosa (ZG), zona fasciculata (ZF) and zona reticularis (ZR), was first recognised by Harley (1858), though it was Arnold (1866) who named the zones. Originally these divisions were made on the basis of morphological differences between cells, but now it is clear that the cells also perform different functions (Tait *et al*, 1980). Fig 1.1 shows a diagrammatic representation of the bovine adrenal cortex (Bravo, 1989).

The zona glomerulosa is the outermost zone of the cortex, adhering to the adrenal capsule. It consists of ball-like cells with relatively small cytoplasmic volume, and characterised by transverse foldings of the cristae of the mitochondria.

The zona fasciculata comprises the bulk of the cortex. The cells of this zone are dispersed in columns and contain abundant lipid deposits. Many more mitochondria are present in these cells than in glomerulosa cells.

The zona reticularis is the innermost zone, adjacent to the medulla, consisting of interconnecting cells of different appearance. Generally they contain less lipid than zona fasciculata cells.

In contrast to the adult human adrenal cortex, the fetal adrenal consists of only two zones, the fetal zone and the definitive zone. It is still not completely clear how full development of the cortex occurs, but a combination of the effects of ACTH and steroid on the immature cells is probably involved (Winter, 1987). Several theories have been proposed to suggest the origins of zonation but the currently accepted view is that cells proliferate both from the zona glomerulosa, and from the 'zona intermedia'

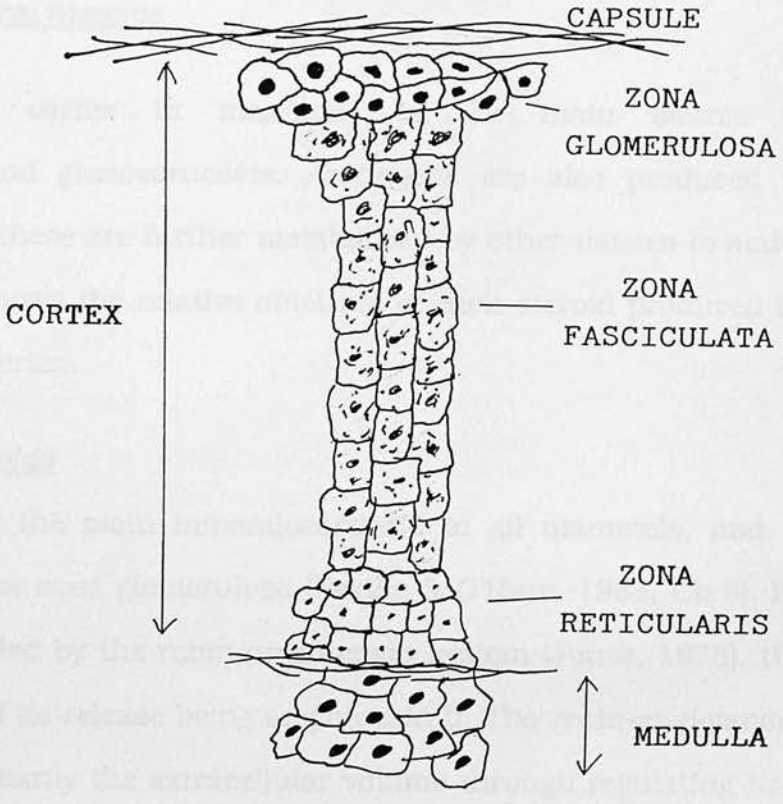


Fig 1.1 Diagram of the bovine adrenal gland (Not to scale).

between the zona glomerulosa and the zona fasciculata (Hyatt, 1987). Hornsby (1987) has proposed that the steroid gradient across the cortex is responsible for affecting cell type and zonation. This theory, though plausible, cannot readily explain the sharp change between the zona glomerulosa and the zona fasciculata.

1.2.4 The Adrenocortical Steroids

The adrenal cortex in mammals is the main source of mineralocorticoids and glucocorticoids. Androgens are also produced in small amounts, and these are further metabolised by other tissues to active hormones. Fig 1.2 shows the relative amounts of each steroid produced by the human adrenal cortex.

1.2.4.1 Mineralocorticoids

Aldosterone is the main mineralocorticoid in all mammals, and is produced solely by the zona glomerulosa (Neville & O'Hare, 1982, Ch 8). Its production is controlled by the renin-angiotensin system (Jones, 1973), the principal stimulant of its release being angiotensin II. The renin-angiotensin system regulates primarily the extracellular volume through regulating Na^+ and H_2O handling by the kidney. Renin production from the kidney is stimulated by several factors, in particular by a reduction in renal perfusion. Renin acts on alpha$_2$-globulin (formed by the liver) releasing angiotensin I, which is in turn converted to angiotensin II by angiotensin converting enzyme, present in the lungs. Angiotensin stimulates aldosterone production from the zona glomerulosa cells of the adrenal cortex. Aldosterone acts primarily on the distal tubule of the kidney to promote Na^+ reabsorption. Na^+ (and an osmotic amount of H_2O) is reabsorbed for an electroneutral exchange of K^+ or H^+.

Steroid	Rate
Cortisol	100
Corticosterone	5
Aldosterone	0.75
11-deoxy-cortisol	3
Deoxy-corticosterone	1.2
Androstenedione	3
Dehydro-epiandrosterone sulphate	125
Dehydro-epiandrosterone	8
Testosterone	0.06
Oestrone	0.02

Fig 1.2 Daily human adult adrenal steroid production rate relative to cortisol (=100) (Normal adult human cortisol production rate = 18-30 mg/day) (Neville & O'Hare, 1982, Ch 8).

Plasma [K^+] also regulates aldosterone production, though this effect is probably of secondary importance when compared to the control exerted by the renin-angiotensin system (Bravo, 1989). ACTH can also promote its production in the zona glomerulosa, but normal aldosterone levels may be maintained in the absence of ACTH (Ganong *et al*, 1968). The importance of ACTH as a physiological regulator of aldosterone levels is still uncertain (Muller, 1988, Ch 4)

1.2.4.2 Glucocorticoids

Glucocorticoids are produced primarily by the cells of the zona fasciculata, but the zona glomerulosa cells are also capable of their synthesis (Tait *et al*, 1980). Cortisol is the main glucocorticoid in most large mammals, including man and cattle, whereas corticosterone is the major glucocorticoid produced by rats, mice and rabbits.

Glucocorticoid production is stimulated by the action of ACTH on the cells of the cortex. The levels of ACTH, and hence cortisol, follow a diurnal rythm, and are also raised during periods of prolonged stress, such as after surgery. ACTH is released from the anterior pituitary by the action of corticotropin releasing hormone (CRH). CRH is derived from the hypothalmus and its release is controlled by innervation. Its secretion is subject to feed-back regulation by endogenous glucocorticoids. Thus the hypothalmo-pituitary-adrenocortical axis controls plasma cortisol levels (Jones, 1973). The production of glucocorticoids from the zona fasciculata / reticularis and their control will be discussed fully in the next section.

Glucocorticoids have a multitude of different effects on the body. In the liver, they promote gluconeogenesis by stimulating the synthesis of gluconeogenic enzymes. This serves to maintain plasma [glucose] for essential body functions, during periods of prolonged stress or in the fasted

state. Glucocorticoids also act on adipose tissue, promoting lipolysis. In other peripheral tissues, such as muscle, they mobilize amino acids by breakdown of protein. The glucogenic amino acids are then transported to the liver to be used in gluconeogenesis. In addition to these metabolic effects, glucocorticoids also have an important anti-inflammatory effect, and act as immuno-suppressants by inhibiting lymphokine release.

At the molecular level steroids generally exert their effect by binding to specific intracellular receptors capable of altering the rate of transcription of DNA. Glucocorticoids act in this way, and a cytosolic receptor has been identified in many tissues by the use of ^{3}H-labelled steroids and recombinant DNA techniques (Gustafsson *et al*, 1987).

1.2.4.3 Androgens

Androgens are produced in small amounts by the cells of the zona reticularis (Winter, 1987; Hyatt, 1987). The major androgens produced are androstenedione and, dehydro-epiandrostenedione. Dehydro-epiandrostenedione sulphate is produced in large amounts in humans (Fig 1.2), but, as a sulphated steroid, it is thought to be inactive (Neville & O'Hare, 1982, Ch 8). Androstenedione and dehydro-epiandrostenedione are metabolised further by other body tissues to estrogens and testosterone depending on the sex of the mammal. It is possible that androgen production plays a part in fetal development of adrenal zonation (Hornsby, 1987). It is not clear what factors are responsible for control of androgen production, but ACTH and a putative androgen stimulating hormone have been suggested as possible candidates (Odell & Parker, 1984-85).

1.3 Cortisol Production and its Control in Zona Fasciculata Cells.

1.3.1 Steroidogenic Pathways

Fig 1.3 shows a diagram of the steroid pathways present in adrenocortical cells (Simpson & Waterman, 1988; Tait *et al*, 1980). The enzymes responsible for adrenocortical steroidogenesis are present in the inner mitochondrial membrane and in the endoplasmic reticulum. They consist mainly of cytochrome P-450 -containing proteins and have linked electron transport systems to provide reducing equivalents (electrons).

The rate determining step of steroid synthesis is the supply of cholesterol to cyt P-450_{SCC} and its conversion to pregnenolone (Jefcoate *et al*, 1986). Cyt P-450_{SCC} resides on the inner face of the mitochondrial membrane and receives electrons from mitochondrial NADPH via adrenodoxin and adrenodoxin reductase. Pregnenolone is then transferred to the endoplasmic reticulum. In humans, sheep, cows and pigs, cyt P-$450_{17alpha}$ is present, which converts pregnenolone to 17-alpha-hydroxy-pregnenolone. A 3-beta-hydroxy-steroid dehydrogenase / isomerase then converts either pregnenolone to progesterone or 17-alpha-hydroxy-pregnenolone to 17-alpha-hydroxy-progesterone. P-450_{C21} then converts these products to the 11-deoxy versions of either corticosterone or cortisol.

11-deoxy-cortisol and 11-deoxy-corticosterone are transported back to the mitochondria where the final reaction, utilising cyt P-450_{11beta}, produces either cortisol or corticosterone respectively. It has been suggested by Jefcoate *et al* (1986) that the relative amounts of final steroid products of adrenocortical cells are regulated by the relative amounts of steroid synthesising enzymes present in each pathway. Several alternative pathways to final steroid products may exist, but it is not completely clear how much

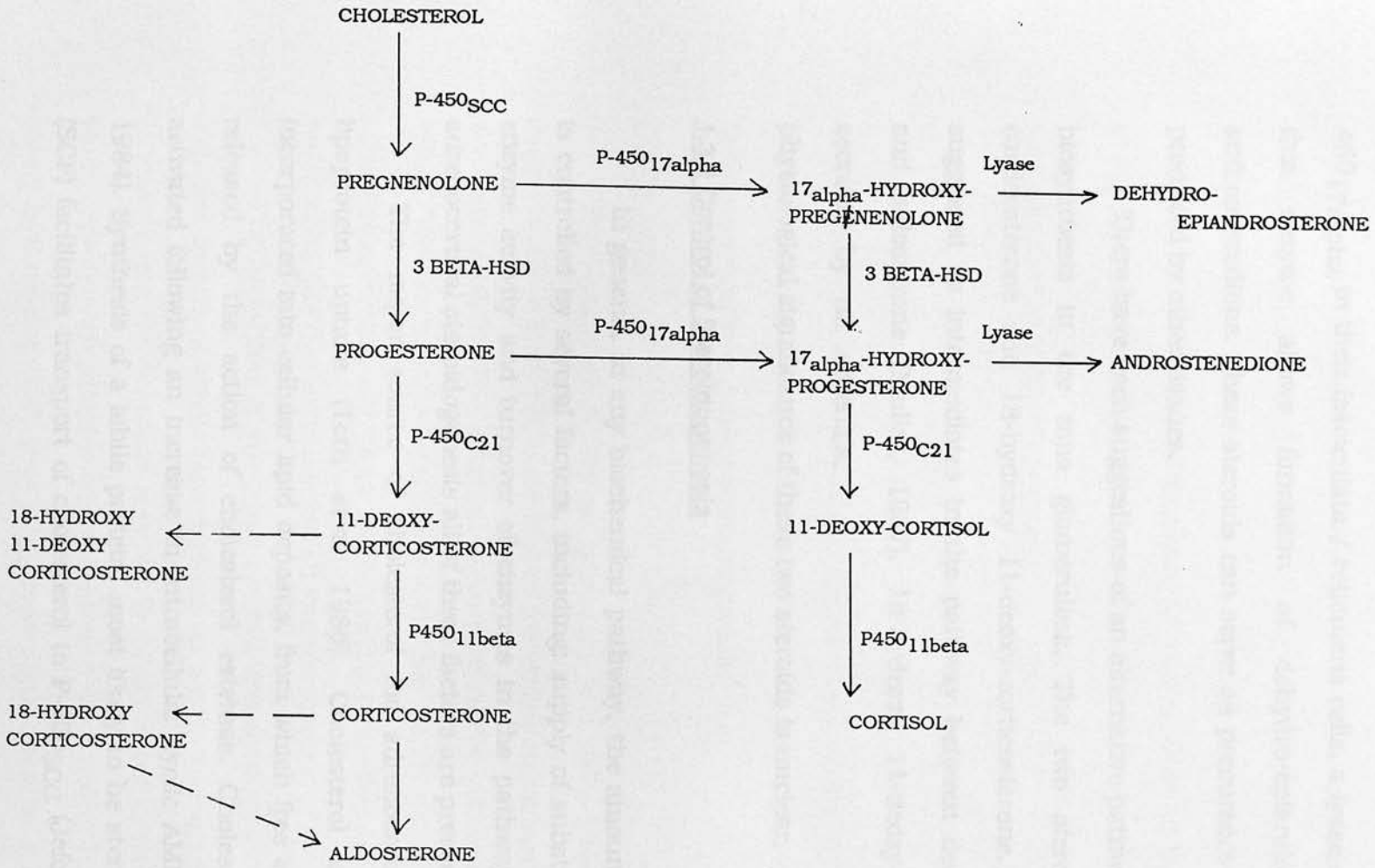


Fig 1.3 Major pathways of steroidogenesis in mammals. Abbreviations are 3 BETA-HSD : 3 BETA Hydroxy-steroid dehydrogenase; Lyase : 17,20-Lyase. (Simpson & Waterman, 1988; Tait *et al*, 1980).

of a contribution they make to overall steroid output (Tait *et al*, 1980).

In glomerulosa cells of all species, corticosterone is converted to aldosterone by an 18-hydroxylation reaction. In species containing P-$450_{17alpha}$ in their fasciculata / reticularis cells, a lyase activity present on this enzyme allows formation of dehydro-epiandrostenedione and androstenedione. These steroids can serve as precursors for sex hormones produced by other tissues.

There have been suggestions of an alternative pathway for aldosterone biosynthesis in the zona glomerulosa. The two steroids, 18-hydroxy-corticosterone and 18-hydroxy 11-deoxy-corticosterone, have both been suggested as intermediates in the pathway between deoxy-corticosterone and aldosterone (Muller, 1987). 18-hydroxy 11-deoxy-corticosterone is secreted by rat adrenals, The exact physiological significance of these two steroids is unclear.

1.3.2 Control of Steroidogenesis

In general, in any biochemical pathway, the amount of final product is controlled by several factors, including: supply of substrate, alteration of enzyme activity and turnover of enzymes in the pathway. In the case of adrenocortical steroidogenesis all of these factors are present.

The major source of cholesterol for adrenocortical cells is via lipoprotein uptake (Toth *et al*, 1986). Cholesterol esters are then incorporated into cellular lipid deposits, from which free cholesterol can be released by the action of cholesterol esterase. Cholesterol esterase is activated following an increase in intracellular cyclic AMP (Vahouny *et al*, 1984). Synthesis of a labile protein, most likely to be sterol carrier protein (SCP) facilitates transport of cholesterol to P-450_{SCC} (Jefcoate *et al*, 1986).

Supply of cholesterol to P-450$_{SCC}$, and its conversion to pregnenolone is the major rate determining step in adrenocortical steroidogenesis.

Longer term control of steroidogenesis is exercised by an increase in steroidogenic enzyme synthesis. This is also probably controlled by increased cellular cyclic AMP. Protein kinase A is activated which phosphorylates specific proteins, leading eventually to increased transcription of mRNA's responsible for coding most of the enzymes shown in Fig 1.3 (Simpson & Waterman, 1988).

1.3.3 Stimulation of Steroidogenesis

Fig 1.4 shows a highly schematic diagram of some of the possible effectors (both positive and negative) of the adrenal cell. Not all effectors are active in all species or zonal cell type. The following sections describe some of these effectors and their mechanism of action, concentrating on their ability to promote or inhibit steroidogenesis.

1.3.3.1 ACTH

The action of ACTH on adrenocortical cells produces the effects described in section 1.3.2, leading to increased steroidogenesis (Simpson & Waterman, 1988). The classical picture of ACTH action on adrenocortical cells - The Sutherland hypothesis - is that ACTH causes an increase in intracellular cyclic AMP which leads directly to steroid synthesis (Grahame-Smith *et al*, 1967). This is seen in all species studied.

The activation of adenylate cyclase and synthesis of cyclic AMP is a common means of signal transduction for many hormones (Rodbell, 1980). In many cases the hormone receptor is linked to the cyclase unit via a GTP binding protein (G protein) which acts as a regulator of hormone action by slowly hydrolysing GTP. It is now known that there is a large family of G

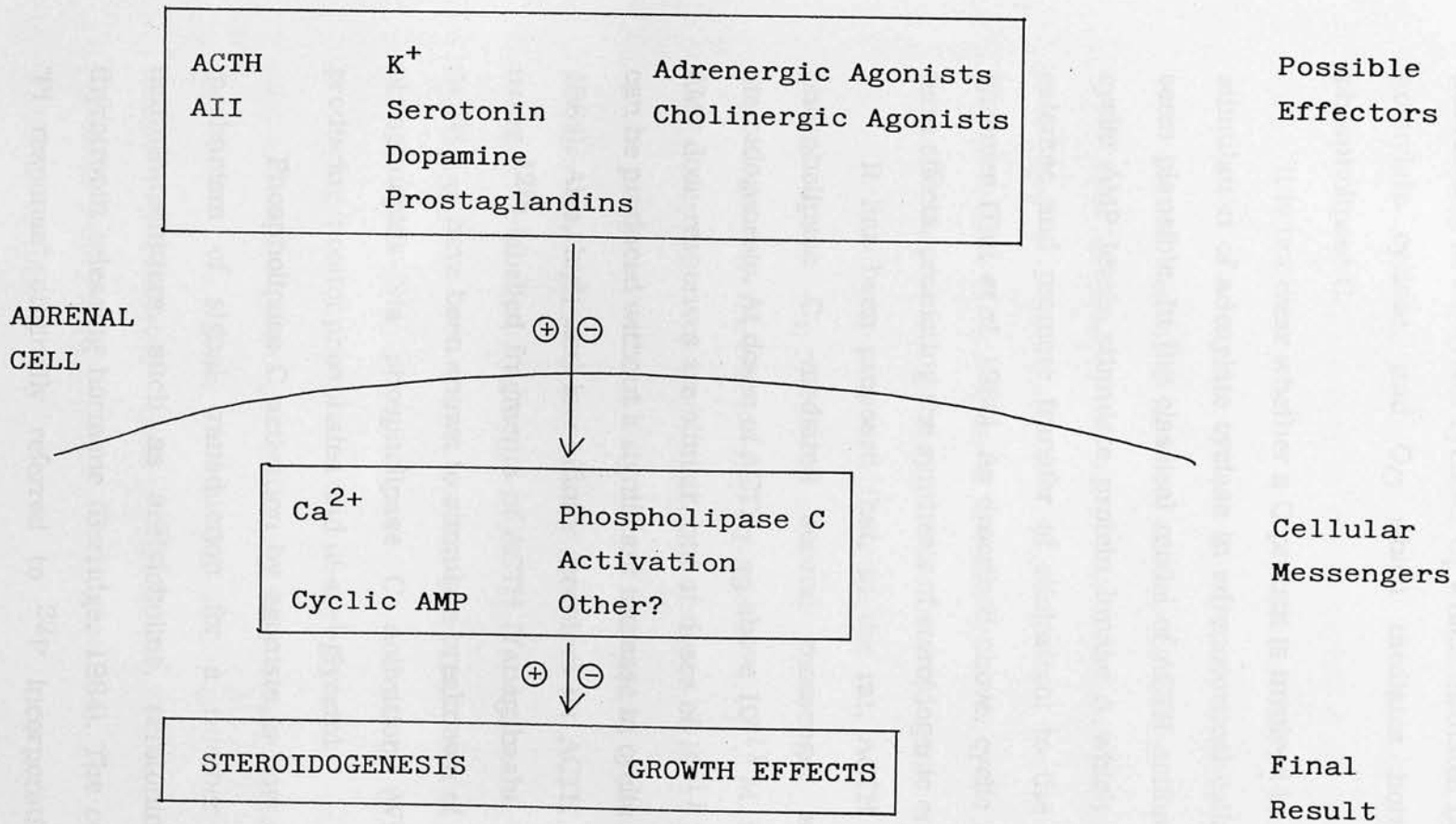


Fig 1.4 Schematic diagram of the major effectors of mammalian adrenocortical cells. Note that not all effectors are active in all species or zonal cell type.

proteins mediating the effects of many agonists (Stryer & Bourne, 1986; Ross, 1989). The three best characterised are: G_S which mediates hormonal stimulation of adenylate cyclase, G_I which mediates hormonal inhibition of adenylate cyclase, and G_O which mediates hormonal activation of phospholipase C.

It is not clear whether a G protein is involved in mediating the ACTH-stimulation of adenylate cyclase in adrenocortical cells, though this would seem plausible. In the classical model of ACTH action, raised intracellular cyclic AMP levels stimulate protein kinase A which activates cholesterol esterase and promote transfer of cholesterol to the steroid synthesising enzymes (Tait *et al*, 1980). As described above, cyclic AMP also has longer term effects, promoting the synthesis of steroidogenic enzymes.

It has been proposed that, in the rat, ACTH may also act via a phospholipase C -mediated second messenger system to promote steroidogenesis. At doses of $ACTH_{1-39}$ above 10^{-11} M, the steroid and cyclic AMP dose-responses are similar, but at doses of 10^{-11} M and below, steroid can be produced without a significant increase in cyclic AMP (Schulster *et al*, 1984). Also, high and low affinity receptors for ACTH have been identified using ^{125}I-labelled fragments of ACTH (Yanagabashi, 1978). Low doses of $ACTH_{1-39}$ have been shown to stimulate breakdown of [^{3}H]-inositol labelled phospholipids via phospholipase C activation (Whitley *et al*, 1987), producing inositol phosphates and di-acyl-glycerol.

Phospholipase C activation by agonists is now a well characterised mechanism of signal transduction for a number of hormones and neurotransmitters, such as acetylcholine, serotonin, vasopressin and thyrotropin releasing hormone (Berridge, 1984). The commonly used term "PI response" originally referred to ^{32}P incorporation into membrane

phospholipids, but is now frequently used (and will be throughout this thesis) to refer to hormonal activation of phospholipase C causing the breakdown of inositol-containing membrane phospholipids to inositol phosphates and di-acyl-glycerol (Fig 1.5). Di-acyl-glycerol can stimulate protein kinase C, leading to phosphorylation of various proteins, while inositol 1,4,5-tris phosphate (the product of the action of phospholipase C on phosphatidyl-inositol 4,5-bis phosphate) is known to cause release of Ca^{2+} from intracellular (non-mitochondrial) stores.

It is possible, in the rat, that ACTH action is mediated by both cyclic AMP and phospholipase C activation, but the precise significance of phospholipase C activation is unclear in relation to the cellular action of ACTH (for review see Bird *et al*, 1990). In the bovine adrenal cortex, there is no reported evidence for low dose effects of ACTH on phospholipid turnover in either glomerulosa or fasciculata / reticularis cells.

There appears to be very little literature on the actual mechanism by which activation of phospholipase C stimulates steroidogenesis. In the currently accepted view of the PI response, phospholipase C cleaves phosphatidylinositol 4,5-bisphosphate to give inositol 1,4,5-tris phosphate and di-acyl glycerol (Berridge, 1984). Inositol 1,4,5-tris phosphate then acts on receptors on the endoplasmic reticulum to release Ca^{2+}, while di-acyl-glycerol activates protein kinase C. In the glomerulosa cell, it is possible that the Ca^{2+} released activates a chain of phosphorylation steps, initially via a calmodulin-dependant protein kinase, and acting finally on the enzymes of the 'early' (cholesterol to pregnenolone) and 'late' (corticosterone to aldosterone) pathways, while protein kinase C acts primarily on the 'early' pathway, stimulating cholesterol to pregnenolone conversion (Quinn & Williams, 1988).

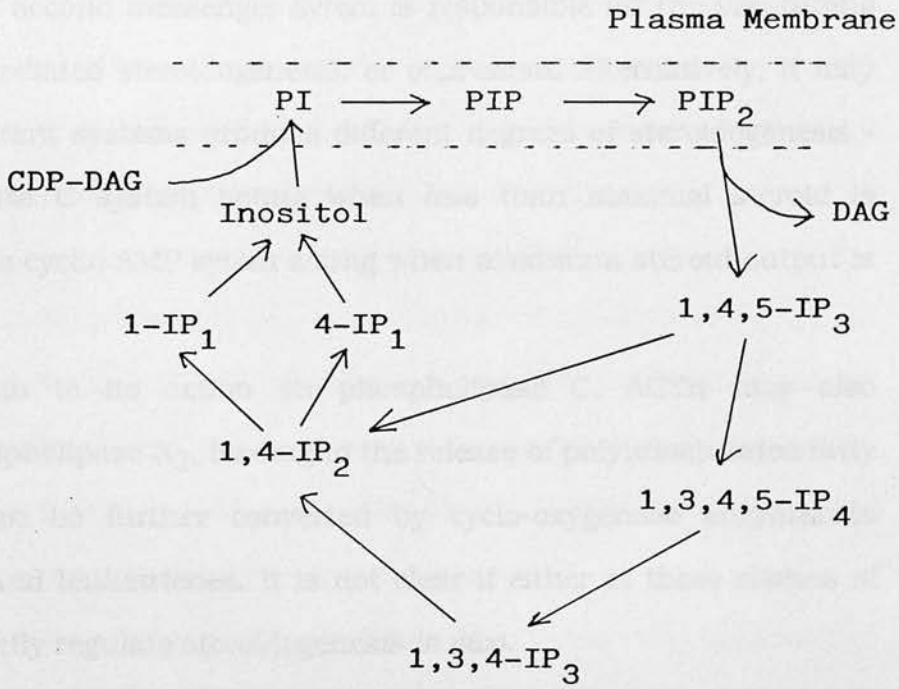


Fig 1.5 The PI response - hormone activation of phospholipase C.
Abbreviations are:

PI	Phosphatidylinositol
PIP	Phosphatidylinositol 4-phosphate
PIP_2	Phosphatidylinositol 4,5-bisphosphate
DAG	Di-acyl-glycerol
CDP-DAG	Cytidine diphosphate - DAG
$1,4,5\text{-}IP_3$	Inositol 1,4,5-tris phosphate
$1,3,4,5\text{-}IP_4$	Inositol 1,3,4,5-tetrakis phosphate
$1,3,4\text{-}IP_3$	Inositol 1,3,4-tris phosphate
$1,4\text{-}IP_2$	Inositol 1,4-di phosphate
$1\text{-}IP_1$	Inositol 1-phosphate
$4\text{-}IP_1$	Inositol 4-phosphate

It is not completely clear why two mechanisms exist capable of mediating steroidogenesis - cyclic AMP and phospholipase C - and how they interact. The mechanisms described above explain *how* each sytem works, but do not explain *why* both exist. One possibility may be that the phospholipase C second messenger sytem is responsible for the fine-tuning of cyclic AMP mediated steroidogenesis, or *vica-versa*. Alternatively, it may be that the different systems produce different degrees of steroidogenesis - the phospholipase C system acting when less than maximal steroid is required, and the cyclic AMP sytem acting when maximum steroid output is needed.

In addition to its action on phospholipase C, ACTH may also stimulate a phospholipase A_2, leading to the release of polyunsaturated fatty acids, which can be further converted by cyclo-oxygenase enzymes to prostaglandins and leukotrienes. It is not clear if either of these classes of compounds directly regulate steroidogenesis *in vivo*.

1.3.3.2 AII

AII is the main stimulant of aldosterone production in rat and bovine glomerulosa cells (Kaplan & Bartter, 1962). While ACTH acts on the early pathway, promoting supply of cholesterol to cyt P-450_{SCC} and its conversion to pregnenolone, AII acts on both the early and the late pathway (Tait *et al*, 1980). In rat glomerulosa cells AII promotes a rise in intracellular [Ca^{2+}] consistent with the involvement of a phospholipase C response (Williams *et al*, 1981). AII has no steroidogenic effect on rat fasciculata / reticularis cells, but bovine fasciculata / reticularis cells do respond, producing cortisol and corticosterone. The only evidence for an effect of AII on glucocorticoid production in the human is from McKenna *et al* (1978) who have shown that in isolated human adrenocortical zona fasciculata /

reticularis cells, AII can stimulate production of cortisol. AII has also been shown to potentiate ACTH-stimulated androgen production from isolated canine adrenocortical cells (Parker *et al*, 1983).

In both bovine and rat fasciculata / reticularis cells, AII produces an increase in phospholipase C -mediated inositol phosphate production, though only in the bovine cells is there a subsequent increase in steroid output (Whitley *et al*, 1987; Bird *et al*, 1989). It has been shown recently that in bovine fasciculata / reticularis cells, phospholipase C is the sole mediator of AII-stimulated cortisol production (Bird *et al*, 1989). The involvement of phospholipase C has been confirmed by Walker, Strachan, Lightly, Williams & Bird (1990) who have shown that AII produces an increase in intracellular Ca^{2+} in bovine zona fasciculata / reticularis cells. Thus AII-stimulated cortisol production from bovine fasciculata / reticularis cells is mediated by stimulation of a phospholipase C.

AII stimulation of phospholipase C in glomerulosa cells is known to involve a G protein which links the activation of the receptor to phospholipase C (Catt *et al*, 1988). There is also evidence to suggest that AII links to a G_I protein in these cells leading to inhibition of adenylate cyclase. In zona fasciculata / reticularis cells it is not known if AII activates phospholipase C via a G protein.

1.3.3.3 K^+, Serotonin and Dopamine

In addition to ACTH and AII, several other hormones, neurotransmitters and ions affect steroidogenesis. Of these, one of the best characterised is K^+. An increase in plasma $[K^+]$ leads to aldosterone production from zona glomerulosa cells (Tait *et al*, 1980). K^+ is thought to be responsible for opening voltage gated Ca^{2+} channels in the plasma membrane (Quinn *et al*, 1988). Hence, both AII and increased extracellular

$[K^+]$ cause an increase in intracellular Ca^{2+}, though the mechanisms appear to differ (Spat *et al*, 1989). It is also possible that elevated extracellular K^+ increases cyclic AMP formation, but this view is not universally accepted (Quinn & Williams, 1988).

Activation of steroidogenesis by AII and raised extracellular $[K^+]$, and their effects on intracellular Ca^{2+} concentration, are complex. In the rat, they appear to cause raised intracellular $[Ca^{2+}]$ in the glomerulosa cells, but to have very little effect on intracellular $[Ca^{2+}]$ in fasciculata cells (Braley *et al*, 1986). Additionally, again in the rat, changes in extracellular $[K^+]$ can modulate the effect of AII action. At low (approx 2mM) $[K^+]$, K^+ inhibits stimulation of aldosterone production by AII (by inhibiting a rise in intracellular $[Ca^{2+}]$). This is thought to be a safety device in the body, preventing aldosterone production and K^+ excretion when plasma $[K^+]$ is low (Barrett *et al*, 1989).

Plasma membrane Ca^{2+} channels appear to be implicated in the action of several, if not all, of the steroidogenic agonists. AII itself and a rise in extracellular $[K^+]$ both open plasma membrane calcium channels in rat glomerulosa cells, but appear to have different effects on intracellular $[Ca^{2+}]$ (Spat *et al*, 1989). This is not unexpected, as there may be direct and indirect effects operating here, as is the case in other cell types. For example, a direct action of the effector (eg. K^+) opening plasma membrane channels and raising intracellular $[Ca^{2+}]$ hence producing steroidogenesis, or an indirect action, where the initial rise in intracellular $[Ca^{2+}]$ is caused by the release of Ca^{2+} from endoplasmic reticulum stores via inositol 1,4,5-tris-phosphate, followed by the opening of plasma membrane channels via inositol 1,3,4,5-tris-phosphate (Irvine & Moor, 1986).

Serotonin and dopamine, both of which are known neurotransmitters,

have also been shown to have effects on steroidogenesis. In the rat, serotonin stimulates aldosterone production from rat glomerulosa cells via a cyclic AMP dependent mechanism (Muller & Ziegler, 1968). Dopamine can inhibit AII-stimulated aldosterone production from bovine glomerulosa cells (Racz *et al*, 1984), but has no effect on ACTH-stimulated aldosterone production (Connell *et al*, 1986). Thus, dopamine may be directly blocking AII receptors or may be interacting with the phospholipase C dependant second messenger system.

1.3.3.4 Adrenergic and Cholinergic Agonists

There is no evidence that K^+, serotonin and dopamine have any effect on steroidogenesis in bovine fasciculata / reticularis cells. However, it has been shown that bovine fasciculata / reticularis cells respond steroidogenically to both adrenergic and cholinergic agonists (Hadjian *et al*, 1981, 1982, 1984; Kawamura *et al*, 1984, 1985). This opens up the possibility that, in addition to the control of steroidogenesis by blood borne secretagogues (ACTH and AII), there may also be a case for innervative control or direct interaction of catecholamines from the medulla with adrenocortical cells. One of the aims of this thesis, stated in section 1.1, is to examine this question and to consider the physiological relevance of adrenergic and cholinergic control of adrenocortical steroidogenesis. Additionally, it is important to investigate the mechanisms of action of adrenergic and cholinergic agonists and to consider how these integrate into the known mechanisms of action of ACTH and AII.

The following two sections (1.4 & 1.5) introduce the adrenergic and cholinergic mechanisms found in mammals, paying particular attention to the receptors and second messenger systems responsible for mediating their effect. Finally, section 1.6 gives a summary of the current literature on

adrenergic- and cholinergic-stimulated steroidogenesis in the bovine adrenal cortex.

1.4 The Adrenergic Response

Adrenaline (epinephrine) and noradrenaline (norepinephrine) are important transmitter substances involved in control of mammalian body function. They can act as localised neurotransmitters in the peripheral or central nervous system, they can have a paracrine function, or, particularly in the case of adrenaline, can act as blood-borne hormones. Noradrenaline acts as the transmitter substance in adrenergic- synapses at the neuromuscular junction of smooth muscles and in the terminal adrenergic- synapses of the sympathetic division of the autonomic nervous system. It also acts as one of the neurotransmitters in the central nervous system (CNS). Catecholamines are also released into the blood by the adrenal medulla. In the human this is mainly adrenaline (80%) with some noradrenaline (20%) (Ungar & Phillips, 1983).

1.4.1 Adrenergic Receptors

At the molecular level, the catecholamines, whether adrenaline, noradrenaline or synthetic derivatives such as isoprenaline, interact with receptors on the cell surface of the target tissue, and, through various means of signal transduction, produce the final measured cellular response.

Research into catecholamines and their effects on cells is extensive and adrenergic mechanisms are some of the best characterised to date. As in all areas of biochemistry and pharmacology, the development of new techniques has been one of the major factors responsible for significant advances in the understanding of the adrenergic response.

Two reviews produced in the last 20 years: by Furchgott in 1972 (Furchgott, 1972) and by Wolfe and Molinoff in 1988 (Wolfe & Molinoff, 1988) provide a comprehensive picture of adrenergic receptors and mechanisms. The review by Furchgott gives the historical perspective of adrenergic receptor classification up to 1972. The requirements set out by Furchgott for adequate receptor classification in this paper are still universally accepted. The review by Wolfe & Molinoff in 1988 summarises the advances in adrenoceptor research between 1972 and 1988, and deals with aspects of receptor regulation. Both of these reviews, however, are outdated by recent advances in gene cloning, in the understanding of the PI response, and in the discovery of further adrenoceptor subtypes.

As early as 1906 Dale (Dale, 1906) showed that ergot alkaloids had different effects on the stimulation of smooth muscle from different organs by adrenaline. This research introduced the use of different agonists and antagonists for distinguishing tissue responses. Langley (1905) had suggested the idea of a "receptive substance" and together with Dale's work established the earliest ideas of receptor classification.

In 1948, Ahlquist (Ahlquist, 1948) looked at the relative potencies of a series of sympathomimetic amines and argued for separate alpha and beta classes of adrenoceptors. The development of synthetic sympathomimetic amines and derivatives of adrenaline and noradrenaline greatly facilitated the clarification of receptor classes. Ahlquist & Levy (1959) used a di-chloro derivative of the synthetic adrenergic agonist isoprenaline to show that the small intestine of the dog contains both alpha and beta-adrenoceptors.

Schild (1947a,b, 1949) produced a significant advance in receptor classification by introducing the idea of pA_2 values for antagonists (see chapter 7 for definition), a means for classifying receptors independent of the

actual effect the drug had on the tissue (its efficacy) and dependent only on its binding characteristics (the affinity). Hence much of the recent work in adrenoceptor and general receptor research has used determination of pA_2 values for selective antagonists as a means of receptor classification.

Furchgott (1960) found a discrepancy in the relative potencies of agonists for the beta-adrenoceptor of rabbit duodenum, and suggested that there may be more than one class of beta-adrenoceptor. In 1967, Furchgott (Furchgott, 1967), again on the evidence of relative potencies of adrenergic agonists and also on the selectivity of adrenergic antagonists, suggested three classes of beta-adrenoceptor and one class of alpha-adrenoceptor. Shortly afterwards, Lands (1967a,b), Arnold & McAuliff (1968,1969) and Arnold & Selberis (1968) firmly established the existence of two classes of beta-adrenoceptor - these were then named $beta_1$ and $beta_2$.

The development of various techniques for attaching radioactive isotopes to drugs and small molecules provided a means for more accurate determination of the binding characteristics of various receptors. In 1979, Minneman (Minneman, 1979) used [^{125}I]-iodo-hydroxy-benzyl-pindolol to show that beta-antagonists varied in their ability to displace the radio-ligand from the two types of beta-adrenoceptor. The radio-ligands: [^{125}I]-iodo-cyano-pindolol, [^{125}I]-iodo-pindolol and [^{3}H]-dihydro-alprenolol are now commonly used for the investigation of beta-adrenoceptors.

In 1984, the development of new beta-adrenoceptor agonists by the pharmaceutical company Beechams Ltd. led to the discovery of a possible third class of beta-adrenoceptor - $beta_3$ - in the brown fat adipocytes of rats (Arch *et al*, 1984). The discrepancy in agonist potency ratios which included the new agonist also suggested the presence of $beta_3$-adrenoceptors in the atria of various mammals (Kaumann, 1989). Although

some workers still dispute its existence, evidence suggests that in certain tissues the effects of adrenergic agonists cannot be explained by two beta-adrenoceptors alone. Physiologically the presence of a third beta-adrenoceptor can be questioned. Adrenaline and noradrenaline are unable to distinguish between a $beta_3$-heart adrenoceptor and a $beta_1$ heart adrenoceptor, so unless a further hormone or neurotransmitter exists or $beta_3$-adrenoceptors operate by a different second messenger system, or are found at different nerve endings, $beta_1$ and $beta_3$-adrenoceptors appear to have identical physiological functions.

The understanding and characterisation of alpha-adrenoceptors has lagged behind that of beta-adrenoceptors, probably because of the lack of suitable specific antagonists in the past, and because the second messengers produced by these agonists were identified at a much later date to those produced by beta-adrenergic agonists.

In 1965, Van Rossum (Van Rossum, 1965) found that the relative potencies of a series of adrenergic agonists on rat vas deferens and rabbit jejunum and the determination of pA_2 values for selective antagonists produced results that suggested the existence of two classes of alpha-receptor. At about the same time Bevan & Osher (1965) also postulated the existence of two classes of alpha-receptor. However, it remained until 1977 for Langer *et al* (1977) to suggest that the pre- and post-synaptic alpha-adrenoceptors were of different types. These were named $alpha_1$ and $alpha_2$.

In 1978, Peroutka (Peroutka *et al*, 1978) used [^{3}H]-clonidine and radio-labelled catecholamines to show that two classes of alpha-adrenoceptor also existed in the CNS, thus supporting Langer's earlier evidence. The alpha-antagonists, prazosin and yohimbine, were beginning to be used at this time to distinguish between alpha-adrenoceptor subtypes.

Prazosin is more potent at alpha$_1$, while yohimbine is more potent at alpha$_2$-adrenoceptors. (U'Pritchard *et al*, 1977; Starke *et al*, 1975).

The situation has become even more complex recently with the suggestion that several classes or subtypes of alpha$_1$ and alpha$_2$-adrenoceptors may exist. Bylund (Bylund, 1988 and references therein) compared the binding characteristics of [^{3}H]-clonidine and [^{3}H]-yohimbine in a variety of tissues to show that at least three subtypes of alpha$_2$-adrenoceptors may exist. This is supported by several other pieces of work (Neylon & Summers, 1985; Michel *et al*, 1989). Functional determination of pA$_2$ values using selective antagonists also appears to support the conclusions obtained with radio-ligand binding studies. Additionally, the alpha$_1$-subclass may be divided into two further subclasses.

With so many new subtypes appearing from different areas of work the adrenoceptor classification scheme has become increasingly complex. How many different types of adrenoceptor really do exist? Both the pharmacological approach of subtyping using agonists and antagonists, and the binding approach using radio-ligands do not give a definitive answer. Gene cloning of adrenoceptors is helping to answer this question. Kobilka *et al* (1987) have cloned two alpha-adrenoceptor genes, and there is evidence that at least four exist. One of the beta-adrenoceptor genes has also been sequenced and the membrane topology of the receptor analysed (Dixon *et al*, 1986; Yarden *et al*, 1986). There are few reports on identification of beta-adrenoceptor subtypes by molecular cloning of the receptor genes, although the beta$_3$-adrenoceptor has been isolated recently by this technique (Emorine *et al*, 1989).

A whole new set of nomenclature is arising from the molecular genetic approach. One of the problems which may emerge is the question of whether

different sequences mean different subtypes or whether this is purely due to species variation. Obviously it is important to match up pharmacological results with those of gene cloning and structure analysis. A combination of methods is likely to lead to a better understanding of the adrenergic receptors.

1.4.2 Adrenergic Receptor Mechanisms

Alpha and beta-adrenoceptors are linked to different signal transduction systems within cells. Both $beta_1$ and $beta_2$-subtypes are coupled via a G_S protein to stimulation of adenylate cyclase (Schulster & Levitzki, 1980, Ch 15)). This process is analogous to the rhodopsin activation of adenylate cyclase in the eye, and both G proteins involved share a high degree of sequence homology (Stryer & Bourne, 1986). It is probable that $beta_3$-adrenoceptors share the same mechanism of signal transduction as $beta_1$ and $beta_2$, because the partial agonist practolol produces both $beta_1$ and $beta_3$ responses (Kaumann, 1989) and stimulates only adenylate cyclase.

Beta-adrenoceptors are subject to homologous, and probably heterologous, regulation mediated by phosphorylation of the receptor, either by a specific beta-adrenoceptor kinase or via another cellular kinase (Leeb-Lundberg, 1987; Huganir & Greengard, 1987). Phosphorylation of the receptor leads to a desensitisation of response to further adrenergic stimuli, and this appears to affect $beta_1$-adrenoceptors to a greater extent than $beta_2$-adrenoceptors (Wolfe & Molinoff, 1988; O'Donnell & Wanstall, 1987).

The different classes of alpha-adrenoceptors appear to be coupled to different signal transduction systems. $Alpha_1$-adrenoceptors are coupled to the activation of phospholipase C (Nichols & Ruffolo, 1988). $Alpha_2$-agonists

produce an inhibition of adenylate cyclase activity (Wolfe & Molinoff, 1988) and may also produce changes in Ca^{2+} metabolism (Nichols & Ruffolo, 1988). Alpha$_1$-adrenoceptors are linked, via the G_o protein, to PI metabolism, while alpha$_2$-adrenoceptors act, via the G_I protein, to inhibit adenylate cyclase activity. There may also be a direct link to membrane Ca^{2+} channels through both of these subtypes.

Differences in second messenger systems coupled to different receptors may provide an answer as to why so many subtypes exist - each individual cell can determine how it responds to an agonist by the proportion of each particular subtype it expresses, thus allowing fine tuning of the response.

1.5 The Cholinergic Response

~~Like noradrenaline,~~ Acetylcholine acts as a neurotransmitter at nerve-nerve synapses - principally in the pre- ~~and post~~-ganglionic synapses of the parasympathetic division of the autonomic nervous system, in the pre-ganglionic synapses of the sympathetic autonomic nervous system, in the CNS and directly on synaptic terminals located on the adrenal medulla (Bowman & Rand, 1984, Ch 9). It also acts as a neuro-transmitter at the neuro-muscular junctions of striated or skeletal muscle.

Dale (1914) discovered that certain effects of acetylcholine on smooth muscle could be mimicked by muscarine and blocked by atropine while some effects of acetylcholine on striated muscle could be mimicked by nicotine. Hence the broad division of cholinergic receptors into muscarinic acetylcholine receptors (mAChRs) and nicotinic acetylcholine receptors (nAChRs) dates from these experiments. This broad subdivision is still in use today.

1.5.1 Muscarinic Receptors

Muscarinic receptors are found in the CNS and at neuro-effector junctions on exocrine secretory glands such as parotid, pancreas and sweat glands, as well as on many smooth muscles including those of the stomach, iris, ileum and gall bladder (Michell, 1980).*

As with adrenoceptors, much of the evidence for cholinergic receptor subtypes has come from the use of specific agonists and antagonists. In the 1970's there was work on mAChRs involving ligand binding experiments using radio-labelled compounds such as quinuclidyl-benzylate (QNB), atropine and scopolamine (Galper *et al*, 1977). However, the first really useful drug used for classification was pirenzipine. Using this antagonist, Goyal & Rattan (1978) showed that mAChRs could be divided into a subclass which had high affinity for pirenzipine (M_1) and a subclass which had low affinity (M_2). Hammer & Giachetti (1982) produced evidence to support this division, but certain groups (Eglen & Whiting, 1985) still debated the existence of two subtypes. One of the main problems was that the evidence for separate subtypes mostly came from studies where pirenzipine had been used in radio-ligand binding experiments whereas very few supportive results existed from functional studies.

Another problem related to the differences which had been observed between ileal and atrial receptors. The antagonist gallamine was shown to be more specific for atrial mAChRs (Mitchelson, 1984) and 4-diphenyl-acetoxy-N-methyl-piperidine methiodide (4DAMP) to be more specific for ileal receptors (Barlow *et al*, 1976).

By 1987 (Levine *et al*, 1987), several subtypes had been postulated. M_1 and M_2 could distinguished by their different affinities for pirenzipine and a third, M_3, receptor had been discovered. The ileal and atrial receptors

* In certain species (not bovine) muscarinic receptors are found on the adrenal medulla.

were also recognised and termed M_2-alpha and M_2-beta respectively. It may be that M_2-alpha receptors can be equated to M_2 and M_2-beta to M_3 receptors, thus simplifying the scheme to three classes of muscarinic receptor. By this stage, mAChR subtyping required the use of at least 5 drugs - pirenzipine, 4DAMP, dicyclorerine, AF-DX116 and hexahydro-sila-difenidol (McN-A-343 was also useful) used in various combinations both in binding experiments and in functional studies (Cohen & Sokolovsky, 1987).

Thus pharmacological analysis has shown the existence of at least three subclasses of mAChRs. From the biochemical point of view the signal transduction mechanisms utilised by these receptors is also of interest. Evidence from both electrophysiological studies (Bolton, 1976) and from investigations of the second messenger systems involved (Birdsall & Hulme, 1976) has shown that, in cells responding to muscarinic agonists, one or more of the following effects may occur (Jarv & Bartfai, 1988):-

(i) Inhibition of adenylate cyclase

(ii) Stimulation of guanylate cyclase

(iii) Stimulation of the PI response

(iv) Influx or efflux of specific cations (eg. K^+)

The question as to which receptor subtype couples to which response has not been fully answered, although the results of gene cloning experiments, to be discussed shortly, are helping to unravel these problems. It has also been suggested that stimulation of PI metabolism is actually the primary event and that all other effects are secondary to this (Michell, 1980) but this appears to be too much of a simplification and it is likely that other mechanisms are also involved (Christie & North, 1987).

1.5.2 Nicotinic Receptors

Nicotinic receptors are located in the CNS, on sympathetic and parasympathetic ganglia, at the neuromuscular junction and on the adrenal medulla (Bowman & Rand, 1984, Ch 9; Ungar & Phillips, 1983). All these receptors appear to be different. Some of the main structural studies on nAChRs have been carried out on Torpedo electric organ (Maelicke, 1988), but again these appear to be different from the mammalian forms.

At the neuromuscular junction of skeletal muscle acetylcholine is released in the synapse and binds to nAChRs on the muscle cells. Tubocurarine is a competitive antagonist of acetylcholine at these receptors.

Using a series of specific antagonists, Paton & Zaimis (1951) first showed that nAChRs at the neuromuscular junction and on autonomic ganglia were different. One of the drugs they studied was suxamethonium, a depolarising nicotinic agonist, which is still used today as a muscle relaxant during certain surgical procedures. Tubocurarine is a naturally occurring, potent antagonist and is the only neuro-muscular blocking drug that has any significant effect on the nAChRs of ganglia/ neuromuscular junction. The alpha-neurotoxins, such as alpha-bungarotoxin, are virtually irreversible antagonists of neuromuscular nAChRs, but have no effect on the nAChRs of ganglia or those in the CNS (Colquhoun *et al*, 1987). Hence there appear to be differences between nAChRs in different tissues, although the evidence for putative subtypes in the literature has not occurred to quite the same extent as for mAChRs. Now that the genes for nAChRs are being sequenced the differences are becoming apparent and these will be discussed shortly.

Most of the research on nAChRs has concentrated on structural studies and electrophysiology. Electrophysiological studies have shown that

and Ca^{2+}

the nicotinic agonists produce an increase in Na^+ and K^+ permeability of the target cells leading to the production of an action potential (Prives, 1980). Patch-clamp and voltage-clamp techniques have been used to show that the nAChR is an ion channel which is opened by the action of a nicotinic agonist (Rang, 1975).

Electron microscopy of the purified receptor from Torpedo electric organ has shown it to be a pentameric channel consisting of five transmembrane domains. Whether this is the case *in vivo* and to what extent the findings apply to the mammalian nicotinic receptor is not yet certain (Changeux, 1987).

1.5.3 Advances in Molecular Biology

Although traditional techniques of pharmacology and biochemistry have provided much information about cholinergic and adrenergic receptors the recent advances in molecular biology have given some of the detailed answers as to how these receptors work.

From sequence analysis and hydropathy studies to determine the extent of protein folding in the plasma membrane, it is probable that there are three or possibly four general families of plasma membrane receptor proteins (Huganir & Greengard, 1987; Schofield & Abbott, 1989; Birdsall, 1989):-

(1) Ligand gated ion channels

(2) Voltage gated ion channels

(3) Receptors that link to G proteins

(4) Receptors that have intrinsic tyrosine kinase activity

Thus, in this scheme, nAChRs fall into category (1) while alpha and beta-adrenoceptors and mAChRs fall into category (3). This model may not

be quite as simple as described, because it may be possible for one receptor to be linked to more than one second messenger system - as could be the case with alpha-adrenoceptors - but it does provide a clear starting point for defining receptor function.

Muscarinic AChRs share a high degree of sequence homology with both beta-adrenoceptors and the visual rhodopsins, all of which have seven trans-membrane domains and link to G protein effector systems (Bonner *et al*, 1987). At least four human mAChRs genes have been isolated and cloned, named HM1 - HM4 by Haga *et al*, 1988, or M_1 - M_4 by Chuan, 1989. Due to problems with nomenclature between groups it is not clear which gene corresponds to which subtype or which signal transduction system it is linked to. Haga *et al* (1988) suggest that HM1 most closely resembles M_1 and HM2 corresponds to the M_2 subtype. HM3 is similar to M_1 and produces inhibition of adenylate cyclase but has no effect on PI metabolism while HM4 is of glandular origin and may be the M_{2alpha} subtype. Chuan (1989) argues, however, that the genes M_1, M_3 & M_4 correspond to the pharmacologically defined M_1 subtype and the M_2 gene to the M_2 subtype. He also suggests that the receptor defined by the M_2 gene couples to adenylate cyclase while M_1, M_2 & M_3 couples to PI turnover.

Cloning studies on the nAChR show that in one species as many as five subtypes (two muscle and three neuronal) may exist (Colquhoun *et al*, 1987). These are simpler than the mAChRs, as they are all ligand-gated ion channels of similar structure with a high degree of homology between species.

Gene cloning has helped to answer some of the problems posed by more traditional pharmacological and biochemical research. The structure, function and diversity of mAChRs & nAChRs is now becoming clearer

* Mikhail & Amin (1969) showed the presence of sympathetic ganglionic cells close to steroid secreting cells from all zones of the human adrenal cortex, and parasympathetic ganglionic cells close to zona fasciculata cells. Migally (1979) showed the presence of autonomic nerve terminals in the zona glomerulosa of the mouse, some of which were associated with blood vessels in the zona fasciculata. Kleitzman & Holzwarth (1985) showed the presence of catecholaminergic nerve terminals in the zona glomerulosa of the rat, associated both with blood vessels and steroid secreting cells. They showed that these terminals were not associated with the splanchnic nerve.

Thus adrenergic innervation could be controlling blood supply to the adrenal (and hence affect steroid secretion by a flow dependant process) and/or may directly influence secretion by catecholaminergic stimulation of cellular steroidogenesis.

through a combination of methods.

1.6 Physiological Relevance of the Adrenergic and Cholinergic Responses in the Adrenal Cortex

1.6.1 Introduction

As described in section 1.3, the classical agonists ACTH and AII have been known to promote steroidogenesis in several species for some time. This is seen in whole animal studies, in perfused whole gland and tissue studies, in enzymically dispersed cells and in culture. In addition, the steroidogenic responsiveness of the cells to these agonists depends on which zone, and from which species, they come. Discovery of possible adrenergic and cholinergic stimulation of the adrenal cortex is less well documented and its physiological relevance correspondingly less clearly defined.

1.6.2 The Adrenergic Response

Positive evidence exists to suggest innervation of the adrenal cortex, both in humans (Mikhail & Amin, 1969), in the mouse (Migally, 1979) and in the rat (Kleitman & Holzwarth, 1985). However, it has proved difficult to demonstrate any *in vivo* effect of catecholamines. No reports appear to exist of innervation, or the lack of it, in the bovine species.

Vogt (1944) observed increased levels of corticosteroid production upon intravenous infusion of adrenaline in cats and dogs, but this was later explained (Pickford & Vogt, 1955) by the stimulatory effect of catecholamines on ACTH production from the anterior pituitary. The efforts of Sandberg *et al* (1953) to show a direct effect of adrenaline also proved fruitless.

In contrast, Inaba *et al* (1975) showed a significant increase in

plasma corticosterone in hypophysectomised rats injected with adrenaline. More recently Edwards and Jones (1987) have looked at the effect of splanchnic nerve stimulation on the adrenal, and provided evidence that this type of stimulation produced steroidogenesis that could not be explained solely by the action of ACTH.

Edwards and Jones (1988) have also shown release of small amounts of CRH from the adrenal medulla in response to splanchnic nerve stimulation, thus suggesting the possibility of a mini CRH-ACTH-glucocorticoid axis (Charlton, 1990). This would require the CRH released to reach the cortex, and there is still some debate as to whether this is possible. (and whether CRH is able to directly stimulate steroidogenesis).

Much of the work on freshly isolated, enzymically dispersed, cells has shown no effect of the catecholamines on steroidogenesis. Sequeira and McKenna (1985) using bovine adrenal zona glomerulosa cells and Kawamura *et al* (1984) in a mixed adrenocortical cell preparation both found no response to catecholamines. DeLean *et al* (1984) also showed no effect of catecholamines on freshly isolated bovine adrenal zona fasciculata cells.

Although the most obvious conclusion from these observations is that there is no direct adrenergic control of steroidogenesis, the evidence supporting adrenergic innervation and the results from primary cultures (to be discussed later) appear to contradict this.

One possible explanation for the failure to observe an adrenergic response in freshly isolated cells is that the enzymic digestion of the adrenocortical tissue, either using trypsin or collagenase, has destroyed any receptors that may have been present.

Shima *et al* (1984) have shown that in an adrenocortical membrane preparation from rat adrenal cortex, adrenoceptors can be detected by the

binding of [^{3}H]di-hydro alprenolol to both capsulated (glomerulosa) and decapsulated (fasciculata / reticularis) portions. They further identified the receptors as being of the $beta_1$-subtype and showed that, in the decapsulated membranes, adenylate cyclase was stimulated by isoprenaline. These membranes were produced from whole tissue, and are therefore not subject to any enzymic digestion.

A further possible explanation for the lack of any response in freshly isolated cells is that catecholamines present in the medulla, after removal of the adrenal gland from the animal, may have produced receptor desensitisation. Increased levels of catecholamines in the highly stressed animal prior to killing may produce this effect.* There is some evidence for the presence of catecholamines in the bovine adrenal cortex after death (Racz *et al*, 1984). Adrenal blood supply is centripetal and after death simple diffusion may lead to significant catecholamine levels in the cortex.

In vivo it is uncertain whether catecholamines released from the medulla could stimulate steroidogenesis in the cortex. It is a well known fact that glucocorticoids from the cortex influence catecholamine synthesis (Ungar & Phillips, 1983; Weinkove & Anderson, 1985), but evidence for the transfer of medullary products to the cortex are few. There has been debate as to the nature of blood supply to the cortex (Vinson *et al*, 1985), yet no clear indication that blood could flow from medulla to cortex. The only possibility appears to be local effects on zona reticularis cells.

After primary culture of several types of freshly isolated adrenocortical cells, the adrenergic response becomes evident: Kawamura *et al* (1984) showed that although a mixed bovine adrenocortical cell preparation produced no response to adrenaline immediately after isolation, the cells would respond after 3 days of primary culture. This was also

* Treatment of animals prior to death with adrenergic antagonists may theoretically stop this effect and may show the presence of an adrenergic response in freshly isolated cells.

reported by DeLean *et al* (1985) in cultures of rat subcapsular cells. DeLean *et al* (1984) also showed that co-culture of rat adrenocortical and medullary cells prevented the cortical cells from developing a response to adrenergic stimulation, supporting the idea that *in vivo*, if catecholamines from the medulla could be transferred to the cortex, they may down-regulate the adrenergic receptors in the cortex.

Appearance of the adrenergic response may thus have the following possible explanations:-

(1) Recovery of cells from the harsh isolation procedure, back to the *in vivo* state.

(2) Resensitisation of adrenergic receptors which had been desensitised by high levels of catecholamines present in the animal prior to, or after, killing.

(3) Appearance of an entirely new response.

Evidence from adrenergic innervation (Mikhail & Amin, 1969; Kleitman & Holzwarth, 1985) mentioned earlier and the presence of beta-adrenoceptors on membranes isolated from rat adrenal cortex (Shima *et al*, 1984) support (1) and (2).

Beta-adrenoceptors have been detected on transformed adrenocortical cells from the rat (Brush *et al*, 1974; Schor *et al*, 1971) and from the human (Hirata *et al*, 1981; Katz *et al*, 1985). Coupling to adenylate cyclase and production of steroid was also seen in cells isolated from some of the tumours. However, in 3 normal human adrenocortical cell preparations studied by Katz *et al* (1985) no response was seen. These observations, in contrast, support the appearance of an abnormal response in the cultured cells, akin to transformation (ie. point (3), above).*

Therefore, though there is evidence for an adrenergic response in the adrenal cortex from studies of innervation of the cortex, radio-ligand binding

* Or support (2) - desensitisation may have occured.

* Robinson *et al* (1977), using a histochemical technique, reported the presence of fine cholinergic fibres in the adrenal cortex, with small cholinergic bodies in the zona reticularis. Aminergic axons were also observed in the outer zones, some associated with blood vessels. Using electron microscopy, Robinson *et al* (1977) also showed axons adjacent to adrenocortical cells.

Cholinergic innervation may be indirectly affecting steroidogenesis by control of blood flow (via innervation of blood vessels) and/or may directly influence secretion by cholinergic stimulation of cellular steroidogenesis.

containing phospholipids) and the cholinergic response was shown to be calcium dependent (Hadjian *et al*, 1984). Kojima *et al* (1986) showed stimulation of aldosterone secretion by acetylcholine in bovine zona glomerulosa cells. They also showed that this was mediated by an increase in phosphoinositide turnover.

There is therefore evidence to suggest that acetylcholine-stimulated steroidogenesis is mediated by some form of increase in inositol-containing phospholipid turnover in both zona glomerulosa and zona fasciculata/reticularis cells.

Cholinergic effects are not limited to the mammalian system. Benjamina *et al* (1987), demonstrated that the cholinergic response of isolated frog adrenocortical cells was mediated by muscarinic receptors. Benjamina *et al* also reported homologous desensitisation of this response.

The first evidence for the effects of cholinergic agonists on adrenocortical cells in primary culture was that of Kawamura *et al* (1985). In a mixed adrenocortical preparation, acetylcholine stimulated production of corticosteroids (measured fluorometrically) in both freshly isolated cells and in cells after 48 hours of primary culture.

Hence, there is strong published evidence for a cholinergic response *in vitro*, with supporting evidence of cholinergic innervation. There is also, evidence for muscarinic receptors on bovine adrenocortical cells and for a cholinergic response mediated by increased phosphoinositide turnover. There is no knowledge, however, of the subtype of muscarinic receptor involved, or of the detailed nature of the PI response in bovine fasciculata /reticularis cells.

1.7 Summary

The current body of published evidence suggests that, in addition to the steroidogenic effect of ACTH and AII on bovine zona fasciculata / reticularis cells, adrenergic and cholinergic agonists may also be involved in some form of modulation of adrenocortical steroidogenesis.

In addition to a full description of the characterisation of a primary culture system for bovine adrenocortical zona fasciculata / reticularis cells, this thesis presents the results of a detailed investigation into the adrenergic response of bovine zona fasciculata / reticularis cells *in vitro*, and the results of a brief study of the cholinergic response of these cells. This allows conclusions to be made about the relevance of adrenergic and cholinergic control of adrenocortical steroidogenesis, and of the mechanisms of action of these agonists.

2 Materials & Methods

2.1 Materials

The materials used in the experiments detailed in this thesis were obtained from the sources listed below. The sources of specific chemicals, reagents and equipment are given beside the appropriate place in the methods section. In addition, general laboratory reagents were obtained from Sigma Chemical Company Ltd. or BDH.

Company Address:-

Aldrich Chemical Company Ltd, The Old Brickyard, New Road, Gillingham, Dorset,SP8 4JL

Amersham International plc, Amersham Place, Little Chalfont, Buckinghamshire, HP7 9NA

BDH, Burnfield Avenue, Thornliebank, Glasgow, G46 7TP

Bio Rad Laboratories Ltd, Caxton Way, Watford Business Park, Watford, Hertfordshire, WD1 8RP

Boehringer Corporation (London) Ltd, Boehringer Mannheim House, Bell Lane, Lewes, East Sussex, BN7 1LG

Canberra Packard Ltd, Brook House, 14 Station Road, Pangbourne, Berkshire, RG8 7DT

Gorgie Abattoir, Edinburgh District Council, 2 Newmarket Road, Edinburgh, EH14 1RH

Hawksley & Sons Ltd, 68 Boston Road, Leicester, LE4 1AW.

Henry Simon Ltd, P.O. Box 31 Stockport, Cheshire, SK3 0RT

ICN Biomedicals Ltd, Lincoln Road, Cressex Industrial Estate, High Wycombe, Buckinghamshire, HP12 4XJ

Immunodiagnostics, Washington, Tyne & Wear.

Jencons, Cherry Court Way Industrial Estate, Stanbridge Road, Leighton Buzzard, Bedfordshire, LU7 8UA.

Lab M, Topley House, P.O. Box 19, Bury, BL9 6AU

LIP (Equipment and Services) Ltd, Dockfield Road, Shipley, West Yorkshire, BD17 7AS

LKB/Pharmacia, Pharmacia House, Midsummer Boulevard, Milton Keynes, MK9 3HP

Lorne Diagnostics, P.O. Box 6, Tywford, Reading, Berkshire, RG10 9NL

Mackay & Lynn Ltd, 2 West Bryson Road, Edinburgh, EH11 1EH

May & Baker Ltd, Liverpool Road, Eccles, Manchester, M30 7RT

MDH Ltd, Walworth Road, Andover, Hampshire, SP10 5AA

Northumbria Biologicals Ltd, South Nelson Industrial Estate, Cramlington, Northumberland, NE23 9HL

Roche Diagnostics Ltd, P.O. Box 8, Welwyn Garden City, Hertfordshire, AL7

Sarstedt Ltd, 68 Boston Road, Beaumont Leys, Leicester, LE4 1AW

Scotlab, Unit 15 Earn Avenue, Righead Industrial Estate, Bellshill, ML4 3JQ

Scottish Antibody Production Unit, Law Hospital, Carluke, Lanarkshire, ML8 5ES

Sigma Chemical Company Ltd, Fancy Road, Poole, Dorset, BH17 7NH

V.A. Howe, 12-14 St. Ann's Crescent, London, SW18 2LS

2.2 Methods

2.2.1 Cell Isolation and Culture

Primary cultures of bovine adrenocortical ZFR cells were established as follows: Bovine adrenal glands were obtained from freshly slaughtered cattle (18 months - 2 years old, Gorgie Abattoir), collected into saline (0.9% w/v) on ice and transported within 20 minutes to the laboratory. All further procedures were carried out sterile (in a class II laminar flow cabinet, MDH Ltd.) and at room temperature unless otherwise stated.

After removal of the surrounding fat, the glands were sectioned into 100um slices using a Stadie-Riggs microtome. The first slice, consisting of capsule and ZG tissue, was discarded and the second slice, consisting of ZFR tissue, was collected into Earle's balanced salt solution (EBS, Northumbria Biologicals Ltd.) containing 0.2% Reagent Grade bovine serum albumin: Fraction V (BSA, ICN Biomedicals Ltd.). Routinely a total weight of 3-5g of tissue slices was used for each primary cell isolation.

After finely chopping the ZFR tissue with scissors, it was digested for 1.5 - 2 hours, at 37^{O}C, in EBS containing 2% (w/v) BSA, 0.1mg/ml DNAase I (Sigma) and 1.5 - 2 mg/ml Worthington collagenase type 1 (Lorne Diagnostics). The tissue was mechanically disrupted by vigorous shaking every 30 minutes. At the end of the incubation, undigested tissue was removed by filtration through 250um nylon gauze (All gauzes, Henry Simon Ltd.) and the cells pelleted by centrifugation in an Heraeus Sepatech Minifuge T (V.A. Howe) at 450g for 20 minutes at room temperature. After resuspension in EBS/BSA the cells were filtered through 100um and then

30um gauze.

Further purification of the ZFR cells was achieved using the column filtration method of McDougall, Williams *et al* (1979), which also served to free the cell suspension from contaminating red blood cells and subcellular debris. Fig 2.1 shows a diagram of the column system described below. Gentle suction using a syringe was used to pull liquid through the column. A scintered glass Allihn funnel (100mm x 20mm disc / 16-40um pore size, BDH) was filled with approx 5ml Sephadex G10 (Sephadex, 40-120um bead size, Sigma) and approx 2ml of Sephadex G50, (100-300um bead size) (both suspended in 0.9% (w/v) saline) layered on top. The column was equilibrated with 15ml of EBS/BSA solution prior to application of the cell suspension. The cell suspension was applied to the top of the column and loaded onto it using gentle suction. The column was then washed with approx 20ml of EBS/BSA. The cells that were trapped between the Sephadex beads were harvested by resuspending the gel (plus trapped cells) in EBS/BSA and then filtering the solution through 30um gauze, which trapped the gel while allowing the cells and EBS/BSA to pass through. Cells prepared in this way were substantially free from debris, red blood cells and clumps of cells.

Cells were centrifuged at 450g/30minutes as before and the resulting pellet resuspended in growth medium. Growth medium was prepared by combining 10% (v/v) fetal calf serum (FCS, Northumbria Biologicals Ltd.) or serum replacement (Various companies, mentioned specifically later), penicillin (50 IU/ml), streptomycin (50 ug/ml), amphoterin B (2.5 ug/ml) in Ham's F10 medium (all additives and Ham's F10 from Northumbria Biologicals Ltd.). The growth medium was warmed to 37^{O}C before use.

After counting the cells using an improved Neubauer haemocytometer

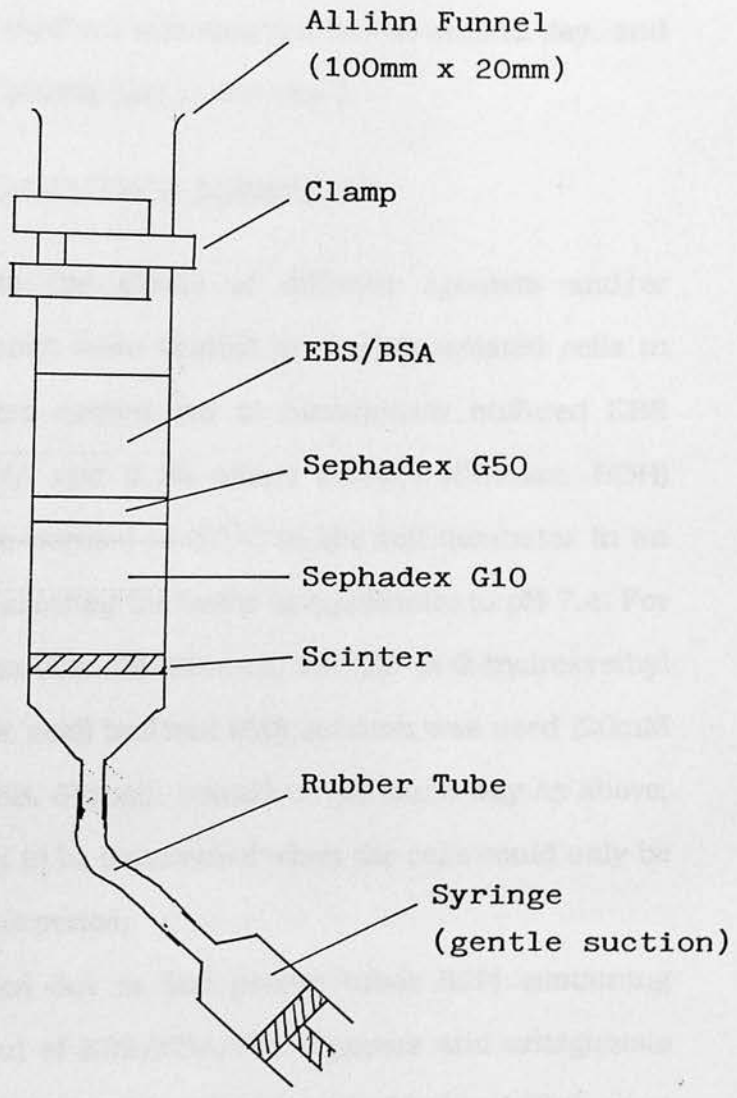


Fig 2.1 Column filtration apparatus used for further purification of the cells (adapted from original method by McDougall, Williams *et al*, 1979.

(Hawksley & Sons Ltd.), they were plated at a density of 250,000 cells/well (1ml/well) in 12 well multiwell cluster plates (Northumbria Biologicals Ltd.) and incubated at 37°C in an atmosphere of 5% CO_2 and 100% humidity (Scotlab VSL incubator)*. The medium was changed on the second day, and the cells used between day of plating (day 1) and day 5.

2.2.2 Agonist/Antagonist Studies on Freshly Isolated Cells

For some experiments the effects of different agonists and/or antagonists on cortisol secretion were studied on freshly isolated cells in suspension. Experiments were carried out in bicarbonate buffered EBS solution containing 0.2% BSA and 0.1% added glucose (Glucose, BDH) (EBS/BSA/Glc). This was pre-warmed to 37°C in the cell incubator in an atmosphere of 5% CO_2, thus allowing the buffer to equilibrate to pH 7.4. For short-term incubations of less than 15 minutes, HEPES (N-2-hydroxyethyl piperazine N'-2-ethanesulfonic acid) buffered EBS solution was used (20mM with respect to HEPES (HEPES, Sigma)), treated in the same way as above. This allowed a more stable pH to be maintained when the cells could only be in the cell incubator for a short period.

Stimulation was carried out in 3ml plastic tubes (LIP) containing approx 250,000 cells in 900ul of EBS/BSA/Glc. Agonists and antagonists were made up in the same EBS/BSA/Glc solution and added in 100ul, or as appropriate, to give a final volume of 1ml. Uncapped tubes were placed at 37°C in the cell incubator. Incubations were terminated by centrifugation of tubes for 15 minutes at 450g in an Heraeus Christ Labofuge 6000 (V.A. Howe). The supernatants were decanted and stored separately at -20°C prior to cortisol assay or immediately acetylated in preparation for cyclic AMP assay. If measurement of intracellular cyclic AMP was required, pellets

* Observation of cells, using phase contrast light microscopy, showed that the cells attached to the cell culture plate between 12-18 hours after initial plating. Initially cells were round, but they adopted a flattened appearance as attachment occured.

were treated with 1ml of 75% ethanol and the sample processed as described in section 2.2.4.

Short term incubations (<15 minutes) were terminated by plunging the bases of the plastic tubes into a methanol/dry-ice bath. The liquid was then thawed as required and treated as above.

2.2.3 Agonist/Antagonist Studies on Cultured Cells

Experiments on cultured cells were carried out in pre-warmed bicarbonate or HEPES buffered EBS/BSA/Glc, depending on the time of incubation, as outlined above.

Routinely, the overlying growth medium was removed from each culture well and the cells were washed (x2) with 1ml of EBS/BSA/Glc. 900ul of EBS/BSA/Glc was added to each well and the experiment initiated by addition of agonists and/or antagonists in 100ul of the same buffer, or as appropriate to give a final volume of 1ml. Where antagonists were used, these were added * before agonists. Plates of cells were incubated at 37^{O}C in an atmosphere of 5% CO_2.

Stimulation was terminated by removal of the overlying liquid into 3.5ml polystyrene tubes and stored at -20^{O}C, for cortisol measurement, or immediately acetylated for cyclic AMP measurement. If intracellular estimation of cyclic AMP was required, cells were extracted by immediate addition of 500ul of ice-cold 75% ethanol. Plates were left for at least 5 minutes before scraping the base with the rubber end on a plunger from a 1ml syringe barrel. Liquid was removed to plastic tubes, a further 500ul of 75% ethanol added to each well, and the washings combined. The extracts were further processed as described in section 2.2.4.

* at least 1 minute

2.2.4 Preparation of Samples for Assay

Cortisol, corticosterone, aldosterone and androstenedione assays could be carried out directly on the cell-free liquid removed from incubations. Routinely the EBS/BSA/Glc containing solution was frozen immediately at -20^{O}C. Prior to assay, samples were thawed and centrifuged in an Heraeus Christ Labofuge 6000 (V.A. Howe) at 4200g for 15 minutes to remove any precipitate which may have formed during storage.

Where cyclic AMP measurement was required, samples were acetylated within 1 hour of the end of an experiment. 500ul of the cell-free liquid removed from the incubations was transferred to a glass tube and acidified to pH 5.0 by the addition of 5ul of 20% (v/v) acetic acid (BDH).

Acetylation was carried out as follows: a 2:1 (v/v) mix of triethylamine and acetic anhydride (both BDH) was prepared in a glass tube, and 15ul added to each sample tube. The contents of the tubes were mixed immediately after addition of the acetylating mixture. These samples were stored frozen at -20^{O}C ready for assay.

Measurement of intracellular cyclic AMP was carried out on cells that had been extracted with 75% ethanol as described above. The extracts were prepared by centrifuging the sample tubes containing the combined ethanol washings for 15min at 4200g in an Heraeus Christ Labofuge 6000 (V.A.Howe). The supernatant was decanted into a glass tube and solvent evaporated under a stream of air while being warmed simultaneously to 37^{O}C on a heating block (Techne Dri-block:DB-3 fitted with sample concentrator:SC-3, Jencons). This process took about 1 - 2 hours. After evaporation to dryness, 500ul of 50mM acetate buffer, pH 5.0, was added to each tube to reconstitute cell extracts, and after mixing to ensure complete solubilisation, acetylation was carried out as described above. Samples were

stored frozen at -20^{O}C until ready for assay.

2.2.5 Radioimmunoassays

2.2.5.1 Cortisol

Cortisol production by the cells was measured by a modified *in-house* radioimmunoassay (RIA), originally developed to determine patient cortisol levels in urine and plasma (Gray *et al*, 1983). The assay employed a double antibody preprecipitate to separate bound from free cortisol.

Cortisol standards were prepared in EBS/BSA/Glc solution from stock cortisol (hydrocortisone, Sigma) - 10mM dissolved in ethanol - and frozen after verification against previous standards. Prepared in this way, standards were stable for up to 3 months. The standard range was 1 - 2000 nmol/l cortisol, with 3 quality control values to check for intra-assay drift and inter-assay precision.

A 0.1M citrate buffer was used throughout the assay, made from citric acid and trisodium citrate (both BDH), pH 4.0, 0.2% gelatine (BDH), 65mg Na azide / litre (azide, Sigma) for storage at 4^{O}C. The second antibody preprecipitate was prepared from 650ul reconstituted sheep anti-cortisol, 530ul normal sheep serum, 15ml donkey anti-sheep (all Scottish Antibody Production Unit), 10ml 0.1M citrate buffer and left overnight at 4^{O}C to equilibrate. This solution was centrifuged, at 230g for 15minutes, and the loose pellet reconstituted in a total volume of 1 litre of 0.1M citrate buffer. Tracer solution was obtained from Amersham as cortisol-3(o-carboxymethyl)oximo-(2-[^{125}I]Iodohistamine) in methanol: water (9:1), approx 2000 Ci/mmol, 0.1 uCi/ul.

The assay was set up with a Clinicon Dilutrend automatic diluter (Boehringer Corporation), using standard 90 tube RIA racks (V.A. Howe),

and 12 x 75 mm polystyrene tubes (LIP). 700ul of 0.1M citrate buffer, pH 4.0, containing approx 10,000 cpm of cortisol tracer, was added to each tube, along with 100ul of either standard solution, quality control or unknown sample. 250ul of preprecipitate was then added to each tube from a constantly stirred stock solution of the same. Using this method, with standards and unknowns in duplicate, placing the standard curve at the beginning of the assay and quality controls throughout the assay, it was possible to set up a maximum of 90 samples in duplicate (12 standards, 6 quality controls and 72 unknowns) without significant intra-assay drift of quality control values. To avoid drift the preprecipitate had to be added in less than 10 minutes to all the tubes.

After mixing the racks of tubes on a multivortex they were incubated in a water bath (37^{O}C) for 70 minutes, centrifuged in an Heraeus Christ Cryofuge 6-4S (V.A. Howe) for 30 minutes at 1800g, and the supernatant decanted.

Pellets were counted for a minimum of 120 seconds on an LKB/Wallac 1261 Multigamma counter linked to a dedicated IBM computer. Calculations were carried out by LKB/Pharmacia RiaCalc program or WHO RIA package, and the standard curve fitted by a 4 parameter binding site (4 parameter logarithmic) model. Intra-assay %CV values were <10% over the working range of the assay and the unknown values falling in this range were considered to be valid. Fig 2.2 (left panel) shows a representative cortisol RIA standard curve.

2.2.5.2 Corticosterone

Corticosterone was measured, as required, in the medium from cell stimulations. The RIA method was essentially that of Al-Dujaili & Edwards (1978), adapted for corticosterone.

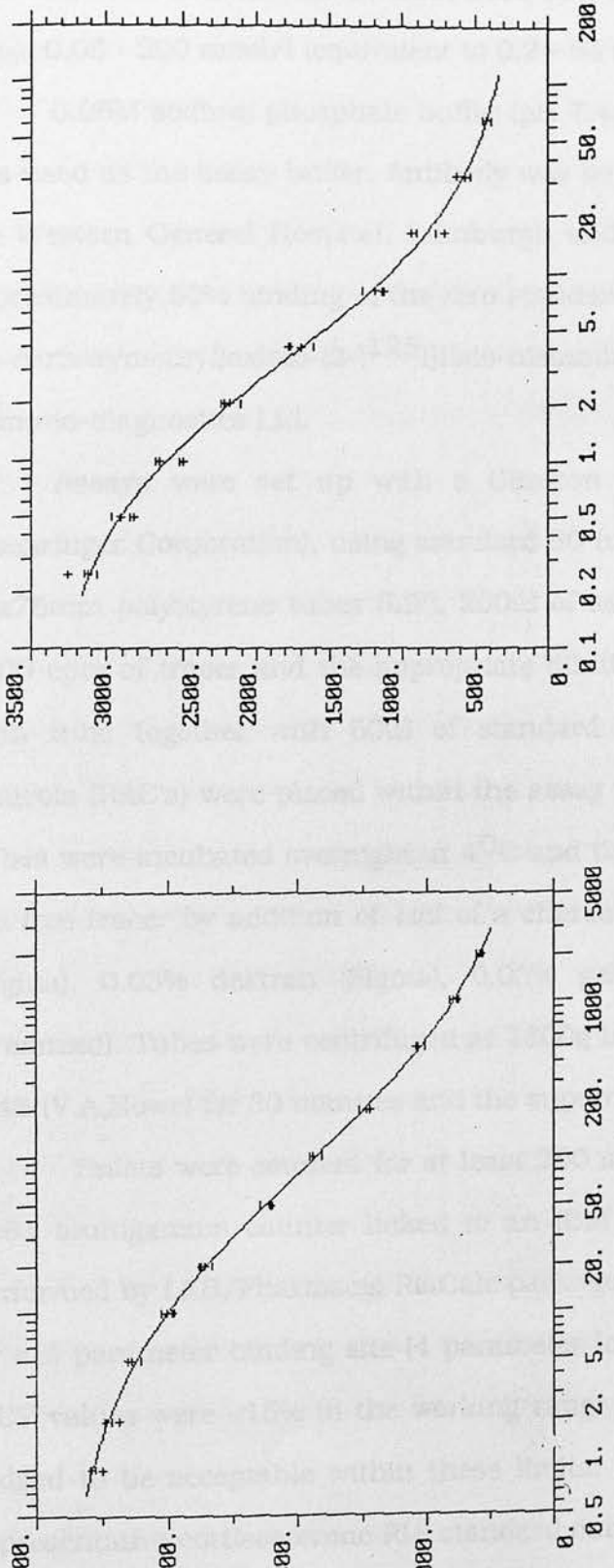


Fig 2.2 *Left panel:* Representative standard curve for cortisol RIA. Four-parameter logarithmic curve fitting used. *Right panel:* Representative standard curve for coricosterone RIA. Four-parameter logarithmic curve fitting used (Original standards, 0.2-64 ng/ml, equivalent to, 0.05-200 nmol/l).

Standard concentrations were prepared from corticosterone (Sigma), and dissolved and diluted in EBS/BSA/Glc. Standard values were in the range 0.05 - 200 nmol/l (equivalent to 0.2 - 64 ng/ml).

0.05M sodium phosphate buffer (pH 7.4, 20^{O}C) containing 0.1% BSA was used as the assay buffer. Antibody was supplied by Dr B.C. Williams of the Western General Hospital, Edinburgh and used at dilutions that gave approximately 50% binding of the zero standard. The tracer, Corticosterone-3(o-carboxymethyl)oximo-(2-[^{125}I]Iodo-histamine), was obtained from Immuno-diagnostics Ltd.

Assays were set up with a Clinicon Dilutrend automatic diluter (Boehringer Corporation), using standard 90 tube RIA racks (V.A.Howe) and 12x75mm polystyrene tubes (LIP). 200ul of assay buffer containing approx 5000 cpm of tracer and the appropriate dilution of antibody was added to each tube together with 50ul of standard or sample. Repeat analysis controls (RAC's) were placed within the assay to check for inter-assay drift. Tubes were incubated overnight at 4^{O}C and then the bound separated from the free tracer by addition of 1ml of a charcoal suspension (0.3% charcoal (Sigma), 0.03% dextran (Sigma), 0.03% gelatin (BDH) in assay buffer (premixed). Tubes were centrifuged at 1800g in an Heraeus Christ Cryofuge 6-4S (V.A.Howe) for 30 minutes and the supernatant carefully decanted.

Pellets were counted for at least 200 seconds in an LKB/Pharmacia 1261 multigamma counter linked to an IBM computer. Calculations were performed by LKB/Pharmacia RiaCalc package and the standard curve fitted by a 4 parameter binding site (4 parameter logarithmic) model. Intra-assay %CV values were <15% in the working range of the assay and values were judged to be acceptable within these limits. Fig 2.2 (right panel) shows a representative corticosterone RIA standard curve.

2.2.5.4 Cyclic AMP

Production of cyclic AMP could be determined either by measuring its appearance in the extracellular medium overlying the cells, or by measuring its intracellular accumulation from ethanol extracts of the cells.

Cyclic AMP was measured by RIA, essentially according to the method of Harper & Brooker (1975). This required the samples and standards to be acetylated to give maximum assay sensitivity. Samples (or standards), tracer and cyclic AMP antibody were incubated together overnight and bound cyclic AMP was separated from free by a second antibody preprecipitate the following day.

The assay buffer used was 50mM acetate buffer, pH 5.0, stored at $4^{O}C$. Standards were prepared in this buffer from a stock solution of 32uM of the TRIS salt of cyclic AMP (stored frozen, Sigma). This stock solution was diluted to give a 320nM standard to determine non-specific binding, and a series of concentrations in the range 0.0625 - 32 nmol/l, for the standard curve. These stock standards were stored at $4^{O}C$ for up to 1 month.

Acetylation of standards was carried out as described previously for sample preparation (section 2.2.4). Acetylated standards were used immediately, or stored at $4^{O}C$ for up to one week.

0.1% BSA (Sigma) was added to the 50mM acetate buffer immediately before use. Rabbit antibody to cyclic AMP was supplied by Dr B.C. Williams of the Western General Hospital, Edinburgh, and was used at a dilution of 1/20,000, judged to give highest sensitivity from its antibody dilution curve. Tracer was obtained from Amersham as adenosine 3',5'-cyclic phosphoric acid 2'-O-succinyl-3-[^{125}I]iodo-tyrosine, approx 2000Ci/mmol, 0.01uCi/ul.

The second antibody preprecipitate used to separate bound from free

cyclic AMP was made as follows - 20ml donkey anti-rabbit antibody and 1.5ml normal rabbit serum (both Scottish Antibody Production Unit) were mixed and left at 4^{O}C overnight. Next day the mixture was centrifuged for 15 minutes at 230g and the loose pellet resuspended in 50ml of 50mM phosphate buffer, pH 7.4. Its ability to precipitate first antibody was estimated by using increasing dilutions of preprecipitate and a concentration used that gave no loss in binding. In general this was of the order of 1:4 to 1:8 relative to undiluted preprecipitate.

The assay was set up with a Clinicon Dilutrend automatic diluter (Boehringer Corporation), using standard 90 tube RIA racks (V.A.Howe), and 12 x 75 mm polystyrene tubes (LIP). 200ul of 50mM acetate buffer, pH 5.0, containing 0.1% BSA, 1/20,000 dilution of cyclic AMP antibody and approx 5,000 cpm of tracer was added to each tube, along with 50ul of either standard or unknown solution. Standards and samples were set up in duplicate, placing the standard curve at the beginning, followed by unknown samples. Repeat analysis controls (RAC's) were placed within the assay to check for inter-assay drift. In general no more than 180 tubes were set up per assay. Tubes were incubated at 4^{O}C overnight.

Next day, 100ul of second antibody preprecipitate was added to each tube, the tubes mixed on a multivortex, and left at room temperature for at least 1 hour. A solution of 1ml of Brij and 500mg micro-crystalline cellulose (both BDH) in 1 litre of water was then prepared and allowed to stir for 15 minutes. 1.5ml of this solution was added to each tube to assist precipitation and act as a wash, and the racks of tubes were centrifuged at 1800g for 30 minutes in an Heraeus Christ Cryofuge 6-4S (V.A. Howe). Supernatant was decanted and radioactivity in the pellet counted for at least 200 seconds in an LKB/Pharmacia 1261 multigamma counter linked to an

IBM computer. Calculations were performed by LKB/Pharmacia RiaCalc package and the standard curve fitted by a smoothed spline. Intra-assay %CV values were <15% for concentrations within the working range of the assay, and values were judged to be acceptable within these limits. Fig 2.3 (left panel) shows a representative cyclic AMP RIA standard curve.

2.2.6 Protein Assay

After cell stimulation, 3 wells of cultured cells or 3 tubes from a freshly isolated cell incubation were kept for protein assay. Culture wells were washed with (2x) 1ml of protein free EBS and 500ul of 1% (v/v) Triton X-100 (BDH) added to solubilise the cells. After 5 minutes, wells were scraped with the rubber end on the plunger from a 1ml syringe barrel and the liquid transferred to a plastic tube. In the case of freshly isolated cells, approx 250,000 cells in a given volume EBS/BSA (in triplicate) were centrifuged for 15 minutes at 250g in an Heraeus Sepatech Minifuge T. These were resuspended in 1ml of protein-free EBS and the procedure repeated, the final pellet being resuspended in 500ul of 1% (v/v) Triton X-100. Finally, for both freshly isolated and cultured cells, the Triton X-100 containing solutions were centrifuged for 15 minutes at 4200g to remove any (non-protein) precipitate and supernatant collected.

Protein was assayed by an automated Bradford assay (Bradford, 1976) adapted for a Cobas Fara centrifugal analyser. Standards (0-100ug/ml) were prepared from a 1mg/ml stock BSA solution in distilled water. These were diluted using 0.1% Triton X-100 to give final standard concentrations in 0.1% Triton X-100.

Samples were diluted 1:10 with distilled water to give a final Triton concentration of 0.1%. A quality control was used to determine inter-assay

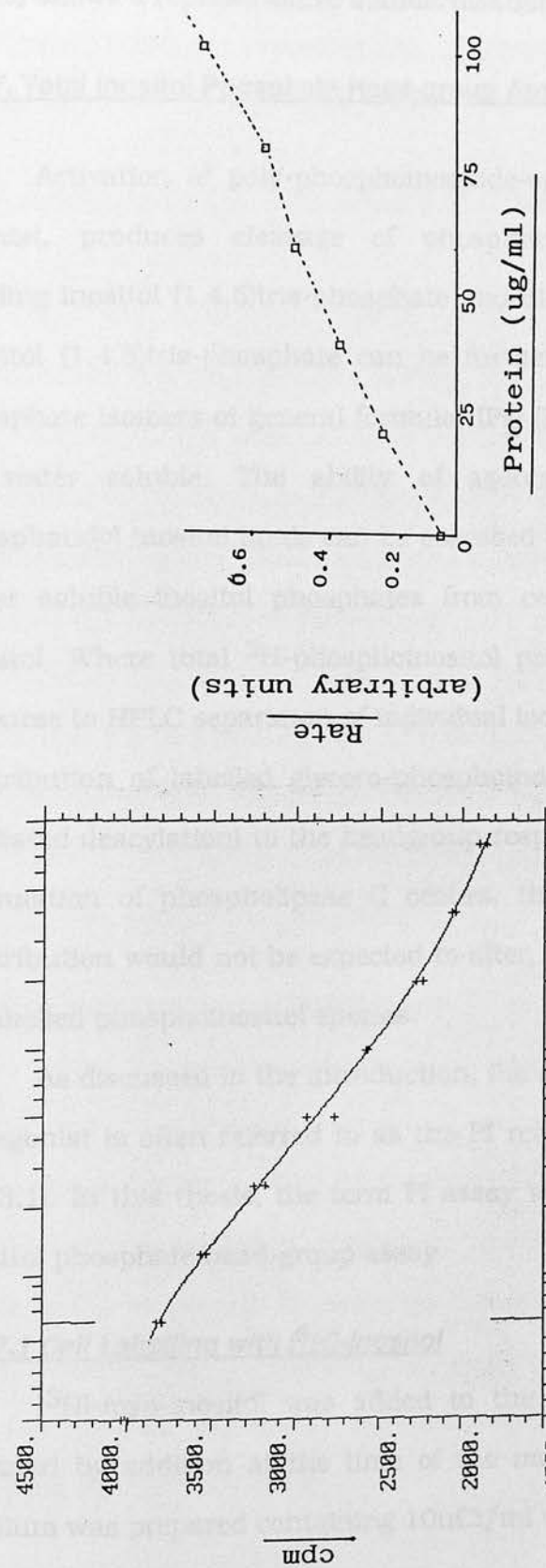


Fig 2.3 *Left panel:* Representative standard curve for cyclic AMP RIA. Smoothed spline curve fitting used. *Right panel:* Standardistaion graph for automated protein assay. Spline fit used (Vertical axis units are arbitrary).

variability and protein was calculated as ug protein/well. Fig 2.3 (right panel) shows a representative standardisation profile.

2.2.7. Total Inositol Phosphate Head-group Assay

Activation of poly-phosphoinositide-specific phospholipase C by an agonist, produces cleavage of phosphatidylinositol 4,5-bis-phosphate, yielding inositol (1,4,5)tris-phosphate and di-acyl-glycerol (Fig 1.5). In turn, inositol (1,4,5)tris-phosphate can be further metabolised to other inositol phosphate isomers of general formula, IP_N (N = 1, 2, 3, 4, 5 or 6), which are all water soluble. The ability of agonists to stimulate turnover of phosphatidyl inositol lipids can be assessed by measuring the production of water soluble inositol phosphates from cells pre-labelled with [^{3}H]myo-inositol. Where total ^{3}H-phosphoinositol production is measured, without recourse to HPLC separation of individual isomers, there will also be a small contribution of labelled glycero-phosphoinositols (from phospholipase A_2-mediated deacylation) to the headgroup response. Where agonist-dependent stimulation of phospholipase C occurs, the small glycerophosphoinositol contribution would not be expected to alter, even with substantial increases in labelled phosphoinositol species.

As discussed in the introduction, the activation of phospholipase C by an agonist is often referred to as the PI response (see definition in section 1.3.3.1). In this thesis, the term PI assay will be used to refer to the total inositol phosphate head-group assay.

2.2.7.1 Cell Labelling with [^{3}H]-Inositol

[^{3}H]-myo-inositol was added to the cells (prepared in the normal manner) by addition at the time of the medium change on day 2. Fresh medium was prepared containing 10uCi/ml of [^{3}H]-myo-inositol (Amersham)

and 0.5ml added to each well for labelling. Previous work had established that isotopic steady state was reached after 42 hours exposure to the label (Bird *et al*, 1989), so measurement of the headgroup response was carried out on day 4 cells (40-48 hours later).

2.2.7.2 Measurement of Total Headgroup Response to Agonist Stimulation

Agonist stimulation experiments on pre-labelled cells were carried out in parallel with unlabelled cells in which cortisol and cyclic AMP could be measured as required, to allow comparison within the same cell preparation.

On day 4 the medium from labelled cells was removed and replaced with 0.5ml of bicarbonate buffered EBS/BSA/Glc (37^{O}C) to wash away any extracellular inositol. After incubation at 37^{o}C for 15 minutes, medium was replaced by 0.45ml of EBS/BSA/Glc (37^{O}C) containing 10mM (unlabelled) myo-inositol (Sigma) and 10mM LiCl (Sigma), and a further 15 minute incubation carried out. At the end of this period additions of agonists or antagonists in 50ul EBS/BSA/Glc was made. After a further 15 minutes incubation at 37^{O}C, the stimulation was terminated by the addition of 250ul ice-cold 15% perchloric acid (BDH).

2.2.7.3 Extraction of Lipid Headgroups

After the addition of perchloric acid, wells were scraped with the rubber end on a plunger from a 1ml syringe and the contents removed to 1.5ml Eppendorf tubes (Lab M). Each well was rinsed with 0.5ml of distilled water and the washings combined. Tubes were then centrifuged in an Heraeus Christ Biofuge B microfuge (V.A.Howe) at 6500rpm for 3 minutes. Supernatants were removed to glass tubes for deacidification by freon:octylamine extraction and neutralisation of perchloric acid. The following method was used:

A fresh solution of 1:1 (v/v) 1,1,2-trichloro-trifluoro-ethane ("freon") and tri-n-octylamine (both Aldrich) was prepared and 1.5ml added to each sample. Samples were mixed thoroughly and tubes centrifuged at 1500g for 3 minutes in a Labofuge 3000 bench top centrifuge (V.A.Howe). Approximately 1ml of the lower phase was removed to waste. A further 1ml of the original extraction mixture was added and the process repeated. Finally 0.9ml of the upper aqueous phase was removed and stored at -20^{O}C.

2.2.7.4 Assay of Total Headgroup Inositol Phosphates

Plastic micro-columns (8x60mm, 70um frit size, lab M) were set up to contain 0.5ml of Bio Rad AG1X8 (200-400 mesh) anion exchange resin (formate form), and the resin washed with 4ml of distilled water.

Samples were thawed, 100ul of 10mM EDTA added to each, and the sample added to one of the columns, followed by 1ml of distilled water to wash the sample container. Each column was washed with a further 2x4ml of distilled water, which was allowed to run to waste (free [^{3}H]-inositol fails to bind under these conditions and is thereby also lost to waste).

Finally, each column was placed over a scintillation vial insert and 2ml of 1M ammonium formate / 0.1M formic acid (both BDH) added to elute the bound phosphoinositols which were collected into the vials. Columns were placed over fresh vials, and the process repeated to ensure complete collection of labelled headgroups. 3ml of Hydroluma scintillation fluid (May & Baker) was added to each vial, and after vigorous shaking, ^{3}H activity was measured in a Packard Tri-carb 300 or 1900CA Scintillation Counter (Canberra Packard). The effects of background ^{3}H-counts and quenching were corrected for automatically.

Total headgroup inositol phosphate production was calculated by combining disintegration per minute values for each pair of scintillation vials

and correcting for volume used in the assay. The measurement of total headgroup production was used as a measure of phospholipase C activity within the cells.

2.2.8 Statistical Methods

2.2.8.1 Calculation of Standard Deviation and the Students 't' Test

In general, all experiments were carried out using triplicate determinations for each agonist and/or antagonist concentration. Figures show mean values obtained (of triplicates, unless stated) and error bars are standard deviations. Where no error bar is shown, the value is too small to be visualised on the graph scale. Experiments were repeated several times (as indicated in figure legends) and figures are representative results or a combination of several experiments (again, as indicated in the figure legends).

The Students 't' test was used to assess significant differences between triplicates within individual experiments. A value of $p<0.05$ was considered to be significant. Figure legends indicate the results obtained, as appropriate.

inappropriate

Care was taken to avoid ~~over~~-use of the students 't' test, particularly as triplicates (n=3) were being compared. When 'n' values are small the assumptions used to derive the equations used for statistical testing break down, and results obtained from them are only of limited value. Additionally, when comparing basal steroid or cyclic AMP output of cells, %CV values for the particular RIAs are high (10-15%) and again statistical tests are of limited value. The students 't' test is used only where appropriate, and generally comparison of results is carried out over a series of similar experiments.

2.2.8.2 Linear Regression Analysis

Where fitting of a straight line to a set of points was required a Casio fx-180P programmable calculator was used. A standard "least squares" equation was used and, where calculation of confidence limits was necessary, the relevant statistical equations and tables were obtained from Bowman & Rand (1984) or Geigy Scientific Tables (8th Edition).

2.2.8.3 Test of Parallelism of Straight Lines

Parallelism of straight lines was tested by analysis of covariance using the SPSS-X statistical package produced by Edinburgh University Computing Service. A value of $p<0.05$ was considered to be significant.

3 Characterisation of Cell Culture I

3.1 Introduction

As discussed in sections 1.2 & 1.3, bovine adrenocortical cells are known to respond to the classical hormonal stimuli of ACTH and AII. This chapter deals with the initial characterisation of the primary cultures of bovine adrenal ZFR cells used throughout this thesis, and their steroidogenic response to ACTH, AII and adrenergic & cholinergic agonists.

3.2 Day by Day Responses of Cells to Agonist Stimulation

Cells prepared as in section 2.2.1 were maintained in primary culture in growth medium prepared using fetal calf serum (FCS). Stimulation of cultured (day 2 - day 5) cells was carried out over a period of 1 hour using $ACTH_{1-24}$ (Synacthen) and AII (section 2.2.3). Freshly isolated, day 1, cells were stimulated in suspension as described in section 2.2.2. Cortisol was measured in the medium (section 2.2.5.1). Fig 3.1 shows representative experimental results of cortisol production over a 1 hour stimulation of day 1 - day 5 cells.

It has also been reported that primary cultures of bovine ZFR cells respond to stimulation with adrenergic and cholinergic agonists (Kawamura *et al*, 1984 & 1985). Experiments were conducted according to the method described above, but using adrenaline and carbachol as agonists. Fig 3.1 also shows representative results of these experiments.

In addition, basal cortisol production was measured over a 1 hour period in unstimulated cells and was shown to be <10 pmol/well on each day. Cellular protein content was assayed on each day over a series of 5

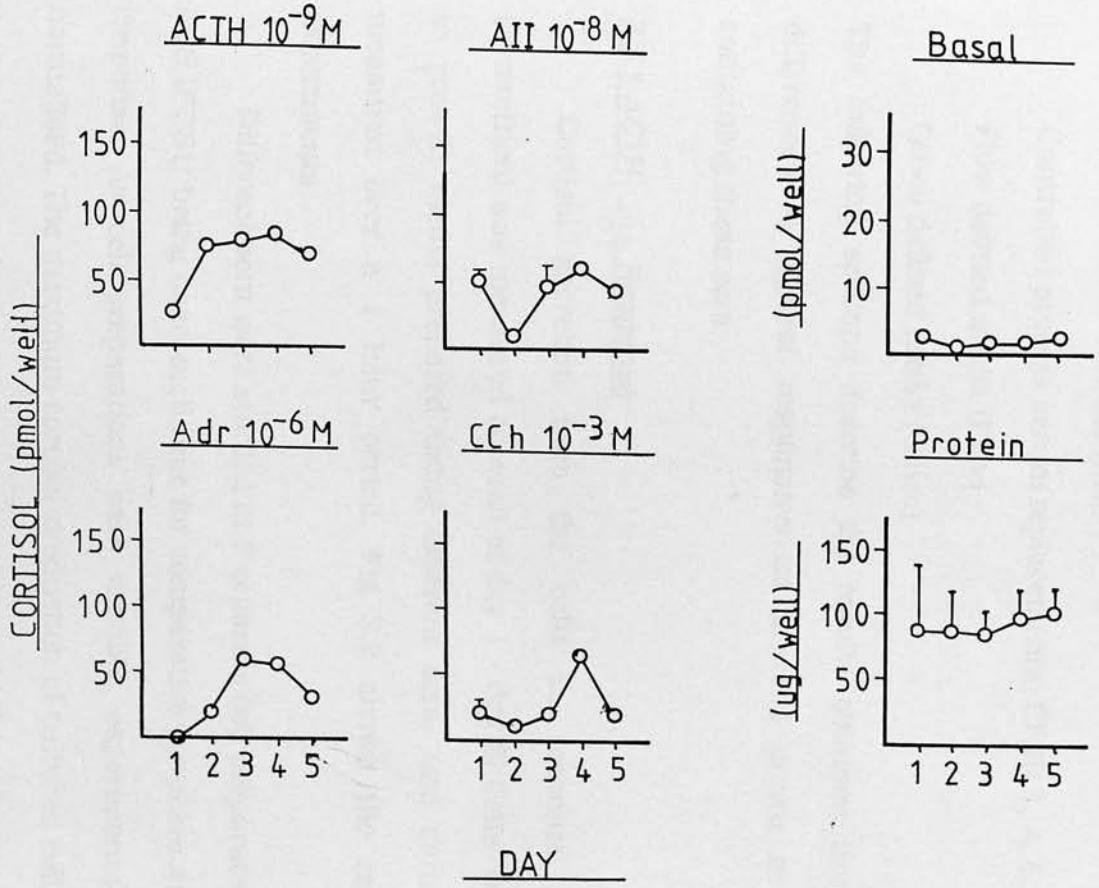


Fig 3.1 Cortisol produced by a 1 hour stimulation of cells with the agonists stated on each of the first 5 days of culture. *Bottom right graph:* protein content of cells on each of first 5 days of culture. Representative experiment (1 of 7 experiments, except protein content results which are the mean values of 5 experiments, n = 15 determinations).

experiments (section 2.2.6) and the combined results are also shown in Fig 3.1.

3.3 Effect of Different Sera on Cultured Cells

FCS was used to prepare growth medium for most of the preliminary experiments. In addition, several commonly available sera were also studied to assess their potential for use in experiments. These were:

FCS - various batches (NBL)

Controlled process serum replacements: CPSR 1, 4, 5 (Sigma)

Flow defined media (Flow)

Gibco defined media (Gibco)

The following sections describe the results obtained from studying the differences in agonist responsiveness of cells grown in growth media containing these sera.

3.3.1 $ACTH_{1-24}$ Response

Cortisol secretion from the cells in response to $ACTH_{1-24}$ (Synacthen) was measured on each of day 1 - day 5. Cells were maintained in growth media prepared using different sera, and cortisol production measured over a 1 hour period. Fig 3.2 shows the results of these experiments.

Different sera were studied in 7 separate cell preparations, a common FCS (FCS1) being used each time for comparative purposes and as a control response. As cell preparations were variable, experimental results were normalised. The maximum cortisol production of cultured cells in FCS1 (day 3 value), in each experiment, was set to 100%. All other values were adjusted accordingly. As FCS1 was used a control in all experiments, this allowed comparison of $ACTH_{1-24}$ responsiveness in different media. Basal

* using a maximal dose of $ACTH_{1-24}$ (10^{-9} M)

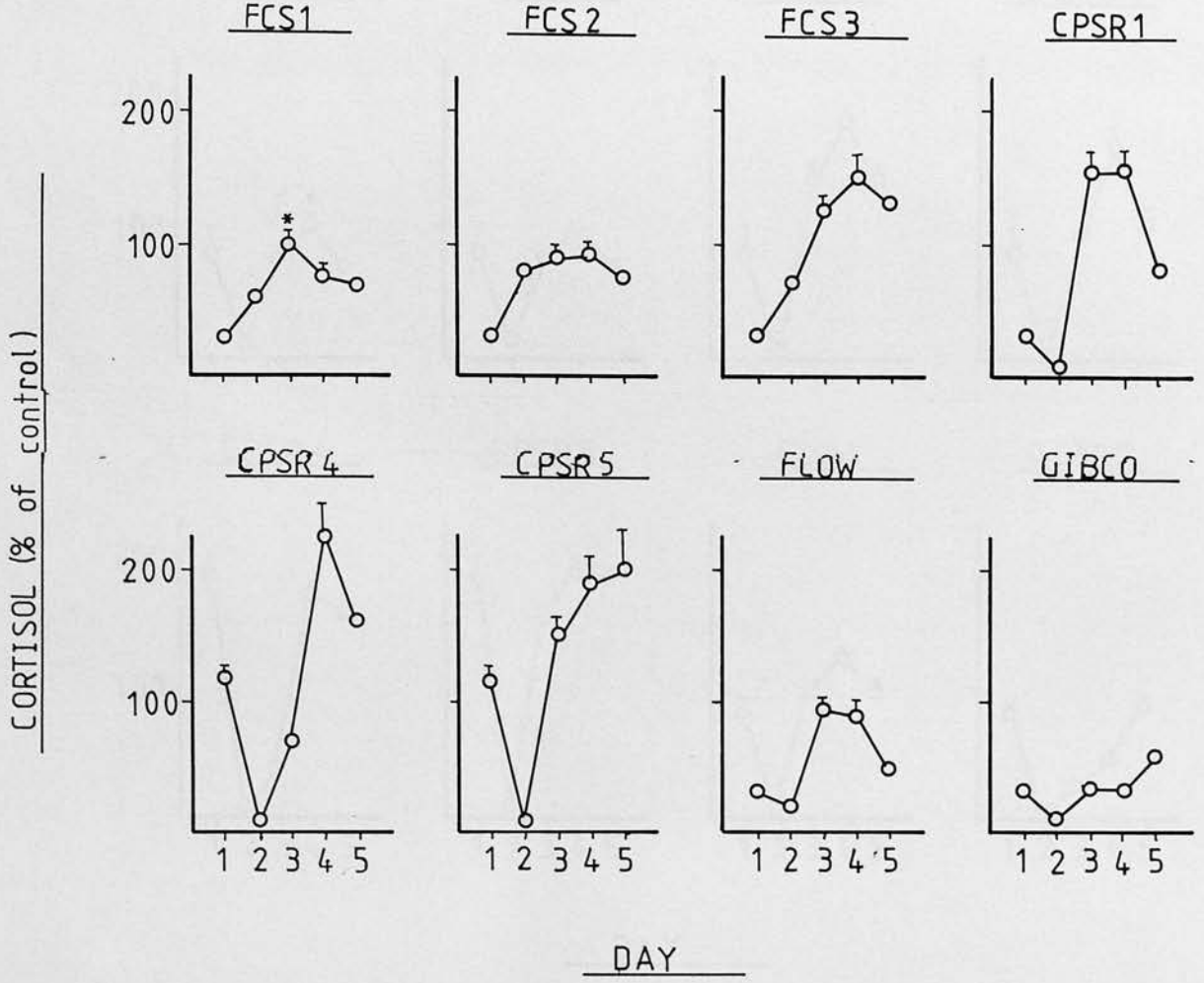


Fig 3.2 Cortisol produced by a 1 hour stimulation of cells with 10^{-9} M $ACTH_{1-24}$ on each of the first 5 days of culture. Reprsentative, normalised (* = 100), results of all media studied (Expt (3) FCS1, FCS2, FCS3, CPSR1 (4) CPSR4, CPSR5, (5) Flow, Gibco; see text).

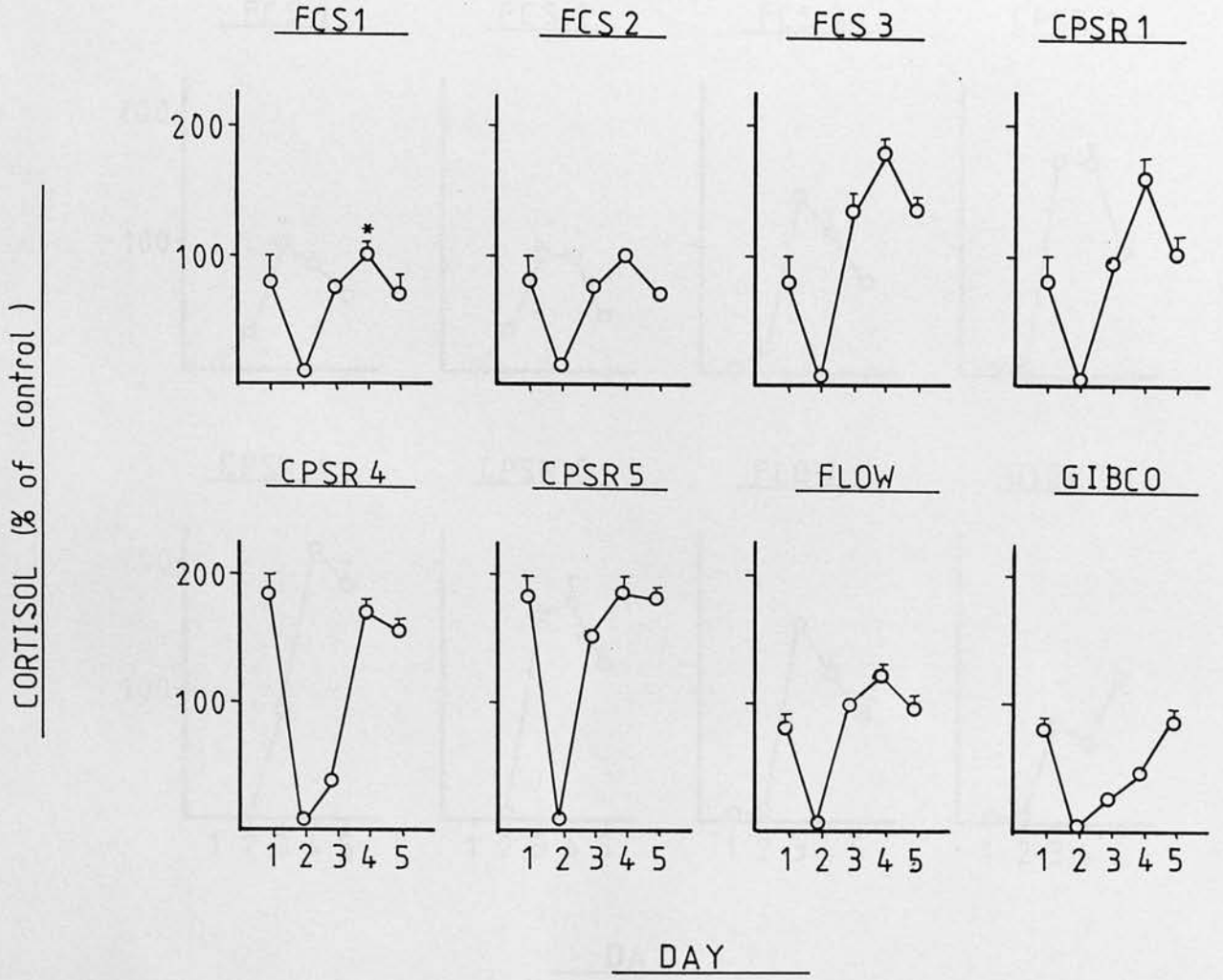


Fig 3.3 Cortisol produced by a 1 hour stimulation of cells with 10^{-8} M AII on each of the first 5 days of culture. Reprsentative, normalised (* = 100), results of all media studied (Expt (3) FCS1, FCS2, FCS3, CPSR1 (4) CPSR4, CPSR5, (5) Flow, Gibco; see text).

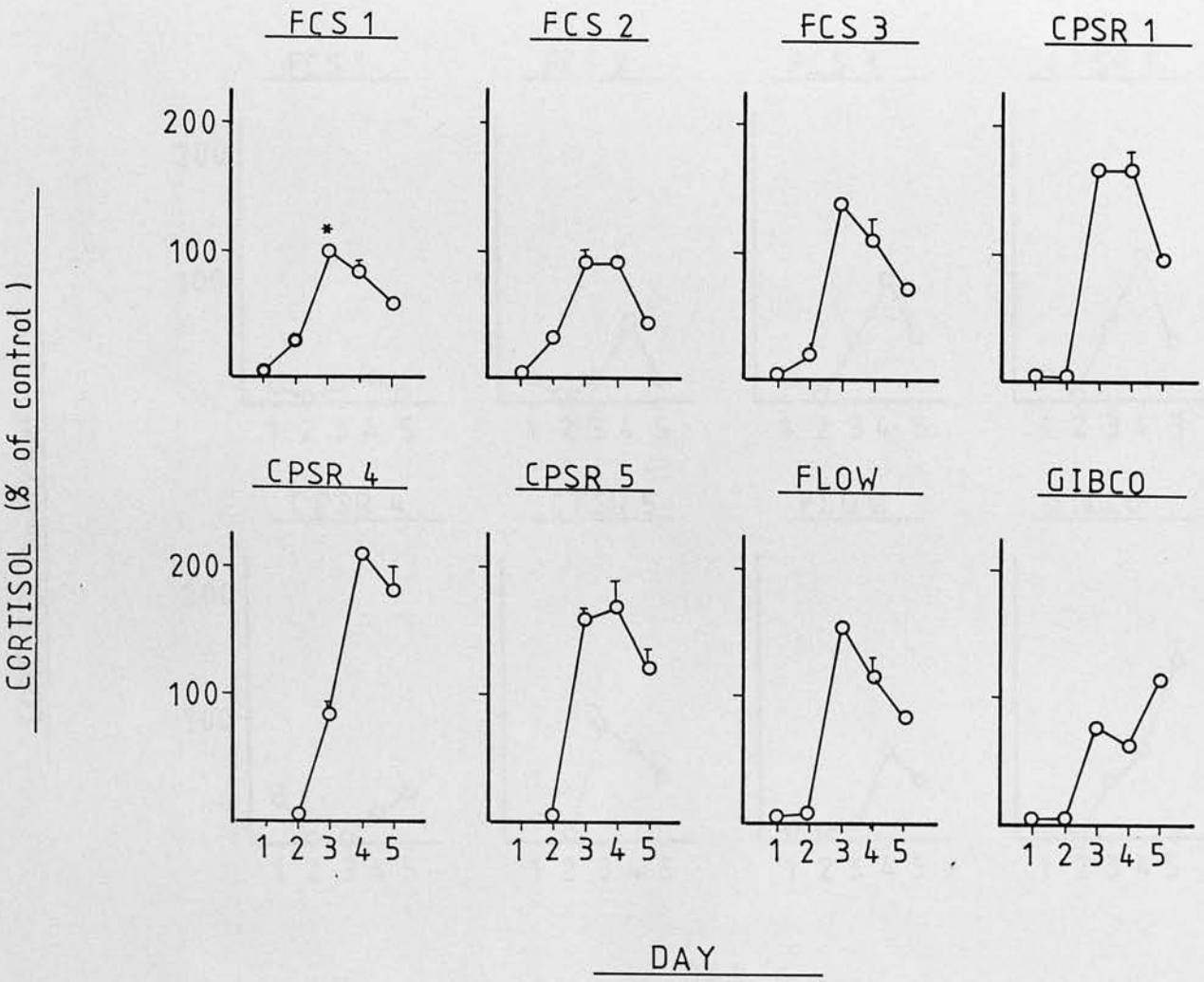


Fig 3.4 Cortisol produced by a 1 hour stimulation of cells with 10^{-6} M Adr on each of the first 5 days of culture. Reprsentative, normalised (* = 100), results of all media studied (Expt (3) FCS1, FCS2, FCS3, CPSR1 (4) CPSR4, CPSR5, (5) Flow, Gibco; see text).

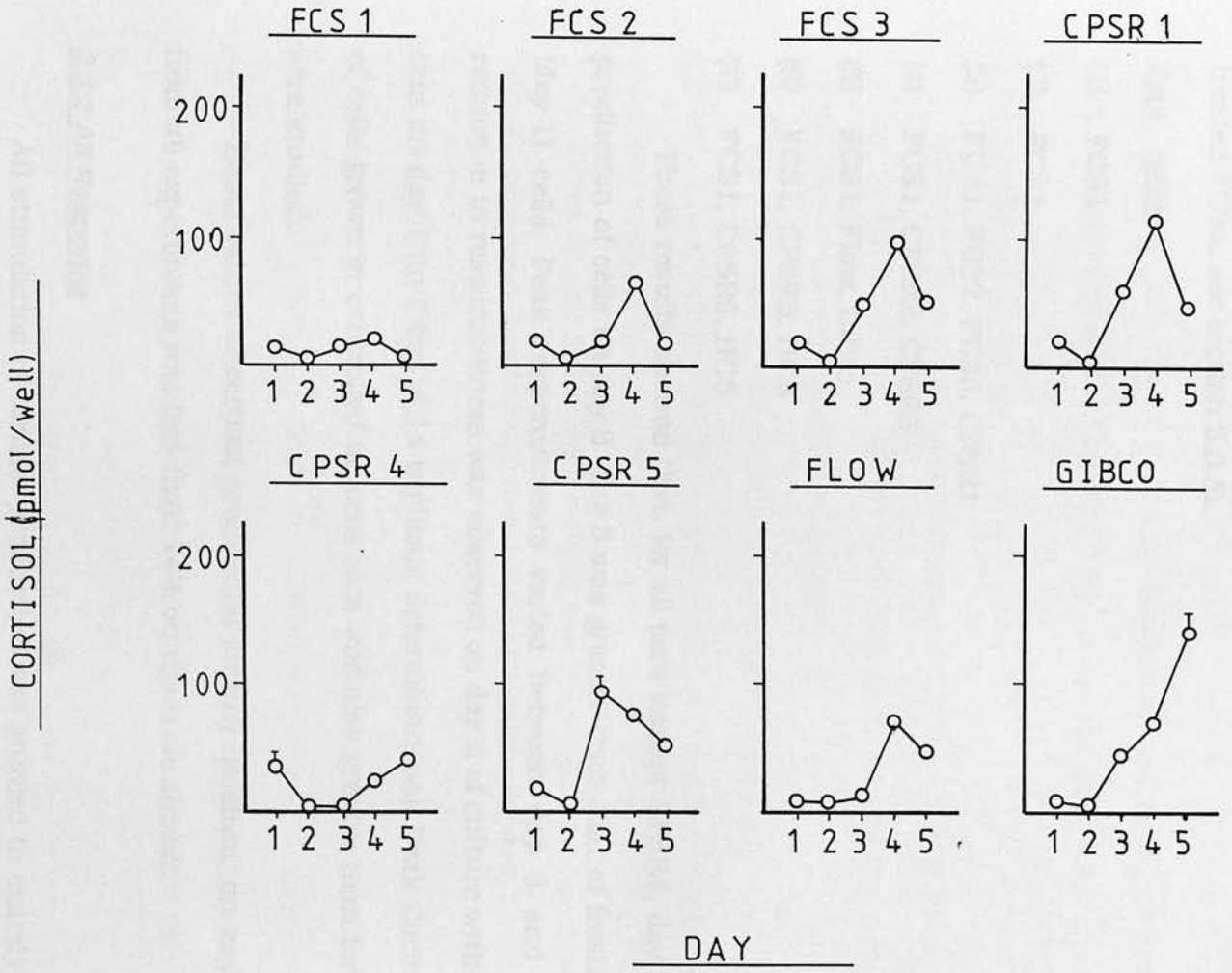


Fig 3.5 Cortisol produced by a 1 hour stimulation of cells with 10^{-3} M CCh on each of the first 5 days of culture. Reprsentative, absolute (pmol/well), results of all media studied (Expt (3) FCS1, FCS2, FCS3, CPSR1 (4) CPSR4, CPSR5, (5) Flow, Gibco; see text).

values were not used to normalise results as, with cortisol levels less than 10 pmol/well (at which point the %CV values for the cortisol RIA precision profiles were high), there were considerable *percentage*, though not *absolute*, differences between the basal values for each experiment. Three separate batches of FCS (NBL), three control process sera (CPSR1, CPSR4, CPSR5 ;all Sigma) and defined sera from Flow and Gibco were studied. In individual experiments, the following growth sera were studied: (HCS = Heat treated FCS1, see section 3.3.5).

Expt Media

(1) FCS1

(2) FCS1

(3) FCS1, FCS2, FCS3, CPSR1

(4) FCS1, CPSR4, CPSR5

(5) FCS1, Flow, Gibco

(6) FCS1, CPSR5, HCS

(7) FCS1, CPSR5, HCS

These results showed that, for all sera (except CPSR4, day 3), cortisol production of cells on day 3 -day 5 was greater than that of freshly isolated (day 1) cells. Peak responsiveness varied between day 3 and day 5. A reduction in responsiveness was observed on day 2 of culture with respect to that on day 1 (in 7 out of 14 triplicate determinations). Peak Cortisol output of cells grown in controlled process sera was also greater than for the other sera studied.

Basal values of cortisol production for any medium, on any day, and from all experiments was less than 14% on the scale shown.

3.3.2 All Response

AII stimulation of cortisol production was studied in exactly the same

* using a maximal dose of AII (10^{-8} M)

manner as for $ACTH_{1-24}$, and data presented in the same format. Fig 3.3 shows the results of these experiments.

It can be seen that, as for $ACTH_{1-24}$, in almost all cases, cultured cell responsiveness was greater than that measured in freshly isolated cells. Maximum responsiveness of cultured cells was again observed to be between day 3 and day 5. The reduction in responsiveness on day 2 was common to all sera, and was observed in all cell preparations (17 out of 17 triplicate determin-ations).

Basal values of cortisol production for any medium, on any day, and from all experiments was less than 14% on the scale shown.

3.3.3 Adrenergic Response

Adrenergic stimulation of cortisol production - using adrenaline - was studied as for $ACTH_{1-24}$ above, and data presented in the same manner. Fig 3.4 shows the results of these experiments.

In the case of adrenaline, cortisol production was not significantly greater than basal in freshly isolated cells. Maximum cellular stimulation was again seen between day 3 and 5.

Basal values of cortisol production for any medium, on any day, and from all experiments was less than 19% on the scale shown.

3.3.4 Cholinergic Response

Cholinergic stimulation of cortisol production - using carbachol - was studied as for $ACTH_{1-24}$ above. Results are presented as amount of cortisol produced in each experiment, and not normalised as for the other agonists because, as will be discussed, the control FCS itself inhibited this response. Fig 3.5 shows the results of these experiments.

The cholinergic response in FCS was found to be very variable. In

$*_1$ using a maximal dose of adrenaline (10^{-6} M)

$*_2$ using a maximal dose of carbachol (10^{-3} M)

some cases cortisol production was only slightly greater in cultured compared to day 1 cells (eg. FCS1), whereas in other cases cortisol production could reach approximately 5 times day 1 values (eg. FCS3). This variability was seen between different batches of FCS and within the same batch. In all experiments freshly isolated cells produced cortisol in response to carbachol (From 1.1 to 34.5 N-fold relative to basal values, 4 experiments). Maximum responsiveness again occurred between day 3 and 5, with a reduction in stimulated cortisol production on day 2 compared to day 1 for all the sera tested (16 out of 16 triplicate determinations).

Basal values of cortisol production for any medium, on any day, and from all experiments was less than 9 pmol/well.

It should be noted that the response of cells to carbachol and acetylcholine produced the same maximum cortisol secretion in all experiments, and these agonists are used interchangeably.

3.3.5 Responsiveness of Cells Grown in Heat Treated Fetal Calf Serum

Consideration was given to the possibility that FCS may contain heat-labile factors detrimental to normal cell agonist responses, and in particular might explain the variable effect of carbachol on the cells.

Heat treated FCS (HCS) was prepared by heating a small volume of serum at 56^{O}C for 1 hour and allowing to cool before being used to prepare growth medium in the normal manner.

Cells were grown in medium containing control fetal calf serum (FCS1), CPSR5 and HCS. Stimulation of the cells was carried out for 1 hour on each of days 1 to 5 using the agonists described above and cortisol measured. Fig 3.6 shows the results of these experiments - presented as absolute cortisol produced.

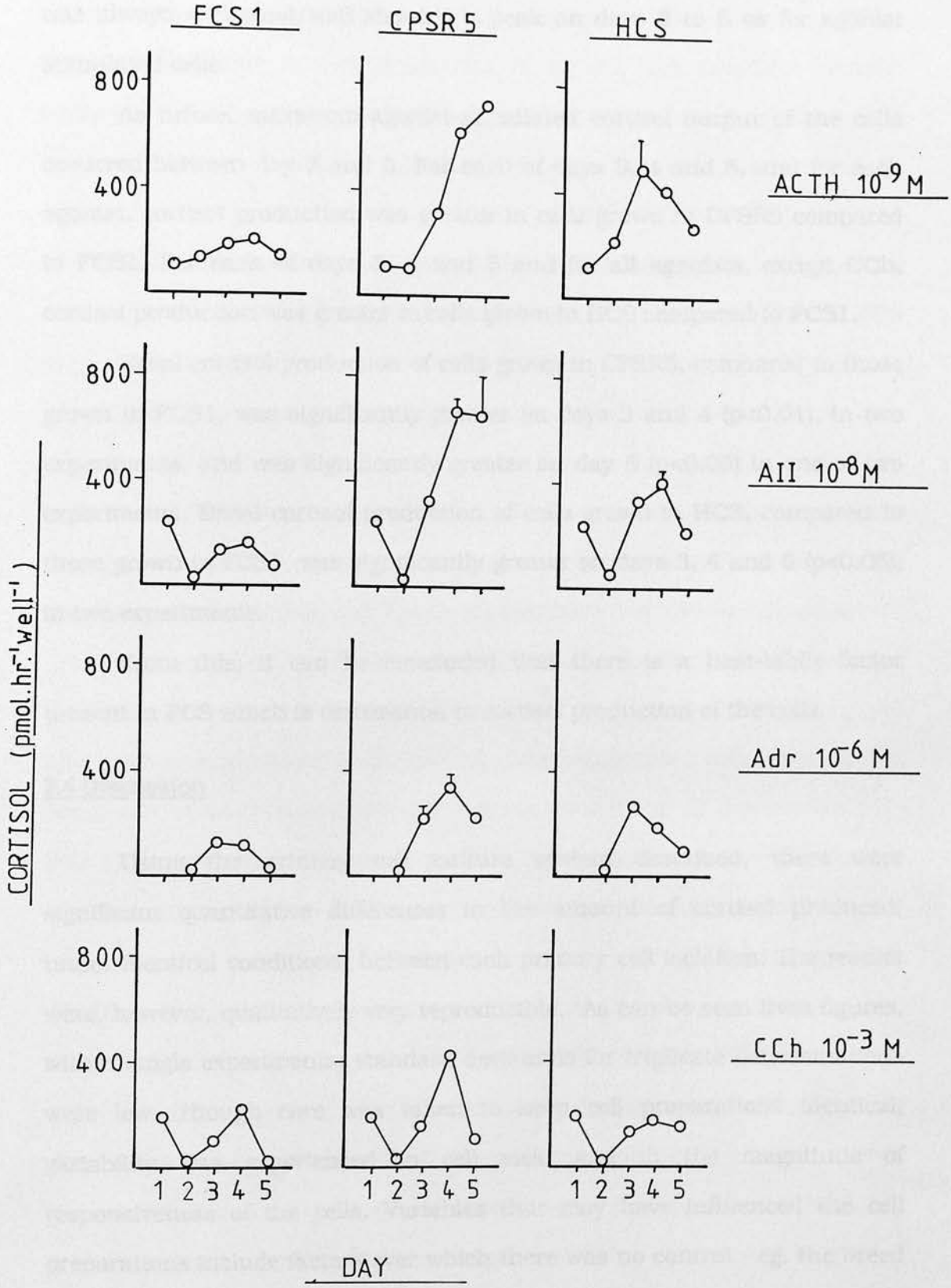


Fig 3.6 Cortisol produced by a 1 hour stimulation of cells with the agonists stated on each of the first 5 days of culture. Representative experiment (Expt (7); see text).

In this experiment, basal cortisol production of cells over days 1 to 5 was always <20 pmol/well showing a peak on days 3 to 5 as for agonist stimulated cells.

As before, maximum agonist-stimulated cortisol output of the cells occurred between day 3 and 5. For each of days 3, 4 and 5, and for each agonist, cortisol production was greater in cells grown in CPSR5 compared to FCS1. For each of days 3, 4 and 5 and for all agonists, except CCh, cortisol production was greater in cells grown in HCS compared to FCS1.

Basal cortisol production of cells grown in CPSR5, compared to those grown in FCS1, was significantly greater on days 3 and 4 ($p<0.01$), in two experiments, and was significantly greater on day 5 ($p<0.05$) in one of two experiments. Basal cortisol production of cells grown in HCS, compared to those grown in FCS1, was significantly greater on days 3, 4 and 5 ($p<0.05$), in two experiments.

From this, it can be concluded that there is a heat-labile factor present in FCS which is detrimental to cortisol production of the cells.

3.4 Discussion

Using the primary cell culture system described, there were significant quantitative differences in the amount of cortisol produced, under identical conditions, between each primary cell isolation. The results were, however, qualitatively very reproducible. As can be seen from figures, within single experiments, standard deviations for triplicate determinations were low. Though care was taken to keep cell preparations identical, variability was experienced in cell yield and in the magnitude of responsiveness of the cells. Variables that may have influenced the cell preparations include factors over which there was no control - eg. the breed

of cattle from which the adrenal tissue was obtained, the age of the cattle, and the effect of any hormones or drugs given to these animals. Other factors, over which a certain amount of control was possible, include: variability of collagenase preparations (for most of the experiments a single stock batch was used) and variability between batches of sera used in culture.

Differences in batches of FCS clearly influenced responsiveness of the cells (eg. Fig 3.2 / $ACTH_{1-24}$ Response). Even using the same control FCS there was variability in responsiveness between different cell preparations which produced quite large statistical error when combining experiments compared to the small errors seen within each experiment. In general, internal controls were used in individual experiments, experiments were repeated at least three times and representative experiments quoted in the results section - a recognised means of presenting data (Su *et al*, 1980).

Occasionally, as in the case of the preceding results section, experiments had to be combined. Results for the agonists $ACTH_{1-24}$, AII and adrenaline were combined by normalising the data: setting the maximum stimulated cultured cell cortisol production of the control FCS (FCS1) in each experiment equal to 100%. This proved to be the best method of presenting results as it allowed comparison of the cultured cell agonist responsiveness between sera. This, however, does not allow true comparison of different agonists in the same serum, and therefore a representative experiment of all the agonists in FCS1 or CPSR5 is given in Fig 3.6.

Results for cholinergic stimulation of cortisol production are not normalised because the results of agonist-stimulated cortisol production in control FCS1 show a high degree of variability. As discussed in section 3.3.5, it may be that heat-labile factors present in FCS are detrimental to normal

cell agonist responses, in particular the cholinergic response. Results for cholinergic stimulation are thus presented as raw data and consequently do not give as fair a comparison between experiments.

It was often observed that there was a drop in cell responsiveness to agonists on day 2 as compared to day 1. This was consistently seen with AII and carbachol but was only seen in 50% of experiments with $ACTH_{1-24}$ (As the day 1 adrenaline response was not significantly above basal in all experiments, day 2 values were always equal or greater than day 1 values).

Observation of cells in culture showed that cell attachment had started to occur by the beginning of day 2: 12-18 hours after initial plating. Observation of cells showed that those grown in sera other than FCS appeared to attach to culture plates more slowly, although this did not appear to significantly affect the peak of cell responsiveness (except possibly in the case of Gibco defined medium which gave a peak for all 4 agonists on day 5). These observations were not measured quantitatively.

As already mentioned, peak responsiveness occurred between day 3 and 5, and, except in case of the Gibco defined medium where this occurred on day 5, cortisol production declined thereafter. Several factors may have affected the timing of the peak response:

(a) Cell plating density: although every effort was made to keep cell density constant, even slight fluctuations may have resulted in differences in the timing of cell attachment to the culture plate.

(b) Availability of lipid: electron microscopic examination of the cells (section 4.1) showed that lipid reserves increased in cultured cells.

(c) Loss of enzyme activity: it is known that activity of certain steroid synthesizing enzymes decreases after several days in culture (Hornsby *et al*, 1979).

(d) Changes in second messenger systems: for $ACTH_{1-24}$ the amount of cyclic AMP produced was greater on day 3 of culture compared to day 1 cells (See chapter 4).

A careful balance between the timing of all these factors probably determines overall daily cellular responsiveness.

In initial experiments FCS was used in the growth medium of cultured cells - most of this work concerned the effect of $ACTH_{1-24}$, AII and adrenaline on the cells. Later investigation of the effect of carbachol showed that the response of the cells in FCS was very variable. From the results presented it was decided to choose a serum that gave a *similar profile of cellular responsiveness* for $ACTH_{1-24}$, AII and adrenaline as for FCS, but an *increased responsiveness to carbachol and acetylcholine*.

Gibco medium was discounted as a choice because peak responsiveness occurred on day 5. Flow medium was not used because carbachol stimulation was very low on the first 3 days. CPSR4 also gave poor carbachol stimulation on days 2 and 3. This left CPSR1 and CPSR5 as possible choices. Both gave similar profiles, with CPSR1 possibly giving higher levels of agonist stimulated cortisol production. However, CPSR5 was chosen because of its higher lipid content (CPSR1 was designed to have a reduced lipid content) so that the bovine adrenocortical cells, specialised with steroid synthesis, had an ample supply of lipid.

Further studies of CPSR5 against control FCS1 (Fig 3.6) showed that HCS gave a much improved cortisol response to all agonists, more like that

obtained with CPSR5 than for FCS1. In particular, the carbachol response was greater, suggesting that heat treatment was destroying one or more inhibitory factors. The basal values of cortisol production in HCS and CPSR5 were also higher, supporting the theory that an inhibitory factor is present in untreated FCS. From the results of agonist effects in the other sera, there appeared to be a general trend that responsiveness of cells in untreated FCS was lower than in the other sera. Heat treatment of sera is carried out to remove factors such as complement proteins which could result in premature cell death through lysis. Though HCS produced good results, because of batch variability in FCS, it was decided to use CPSR5 as a more carefully defined replacement.

To summarise, most of the earlier experiments on steroidogenesis produced by $ACTH_{1-24}$, AII and adrenergic agonists were carried out in FCS, allowing comparison with similar conditions used in published literature. Later experiments, and all those involving cholinergic agonists, were carried out in CPSR5. Appendix I contains details of the serum used in individual experiments presented in figures.

4 Characterisation of Cell Culture II

4.1 Introduction

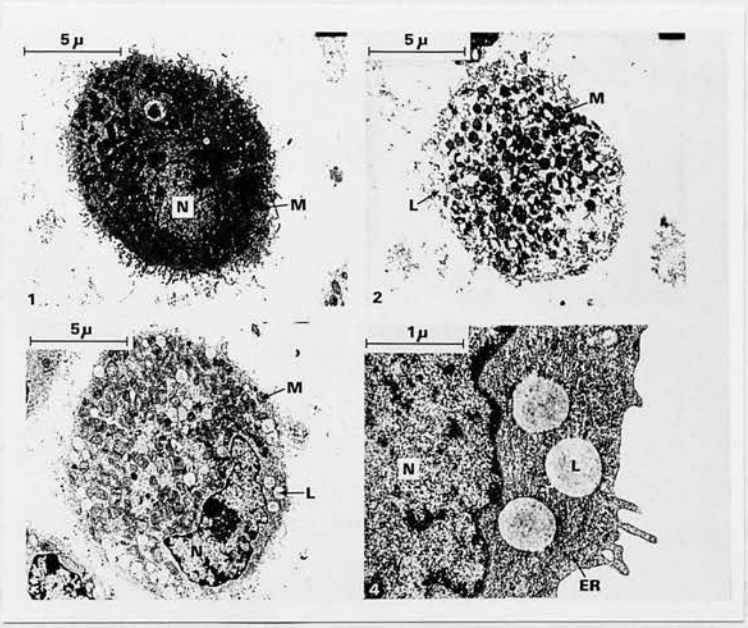
The previous chapter defined the optimum serum requirements for studying the agonist responses of bovine ZFR cells, and the daily responsiveness of the cells. This chapter deals with experiments designed to compare, in more detail, the effects of agonists on uncultured (day 1) cells and on cultured (days 3 & 4) cells and to look at the time-courses of steroid and second messenger production.

4.2 Morphological Analysis of Cells

Light microscopy of freshly isolated (day 1) cells and those on day 3 of culture confirmed that the cultured cells had attached to the surface of the culture plate, adopting a flattened, elongated appearance relative to the rounded appearance of day 1 cells.

For electron microscopy, cell pellets from day 1 and day 3 cells were fixed in 2.5% (v/v) glutaraldehyde in cacodylate buffer (0.1M, pH 7.3). Osmium tetroxide (1% in cacodylate buffer) was used as a secondary fixative. The fixed samples were dehydrated through graded alcohols into propylene oxide and finally embedded in Araldite resin. Ultrathin sections (50-60um) were stained with uranyl acetate and lead citrate in preparation for photomicrography using a Philips EM300 electron microscope. Approximately 100 cells were studied in at least 3 different ultrathin sections from each cell preparation.

Electron microscopy of the cells showed that cultured (day 3) cells had good integrity of ultrastructure and a well-defined plasma membrane



a	b
c	d

Plate 4.1 Ultrastructural comparison of day 1 and day 3 cells: electron micrographs. N = nucleus, M = mitochondria, L = lipid, ER = endoplasmic reticulum. Scale bars shown in um.
(a) Typical day 1 cell.
(b) Damaged day 1 cell, showing indistinct plasma membrane.
(c) Typical day 3 cell, showing numerous lipid droplets (L).
(d) Portion of cell shown in (c), showing good integrity of plasma membrane and abundant enoplasmic reticulum (ER).

(Plate 4.1c,d). In contrast, freshly isolated cells had a more ragged appearance, of which a small percentage (10-15%) were damaged (Plate 4.1a,b). Lipid droplets (L) could be seen in all 4 electron micrographs (Plate 4.1a,b,c,d).

To evaluate the extent of the difference in lipid content between freshly isolated cells and cultured (day 3) cells, the number of lipid droplets per cell section were counted by light microscopy in thick sections (2-3um) stained with toluidine blue. In freshly isolated cells, 4 lipid droplets per cell section (n=100 cell sections, SD=1) were counted, compared with 25 droplets per cell section (n=100 cell sections, SD=2) for the cells on day 3 of culture.

4.3 Comparison of Cultured and Freshly Isolated Cells

4.3.1 Cortisol Production

Dose-response experiments to $ACTH_{1-24}$, AII and noradrenaline were set up with freshly isolated (day 1) cells and cultured (day 3) cells from the same cell preparation. Cells were stimulated for 1 hour as described in sections 2.2.2 & 2.2.3 and cortisol measured (Fig 4.1).

In 6 out of 7 experiments basal cortisol production was greater on day 3 than day 1 ($p<0.05$). $ACTH_{1-24}$ at 10^{-9} M gave a maximum stimulation, and in 6 out of 6 experiments this was greater on day 3 compared to day 1 ($p<0.01$). Likewise, a maximum stimulatory dose of AII at 10^{-8} M gave a greater response in day 3 cells than with day 1 cells in 5 out of 5 experiments ($p<0.01$). In the case of noradrenaline, there was no significant stimulation of cortisol secretion at any dose used in the freshly isolated cells, whereas a highly significant stimulation was observed in cells on day 3 ($p<0.001$ for 4 out of 4 experiments).

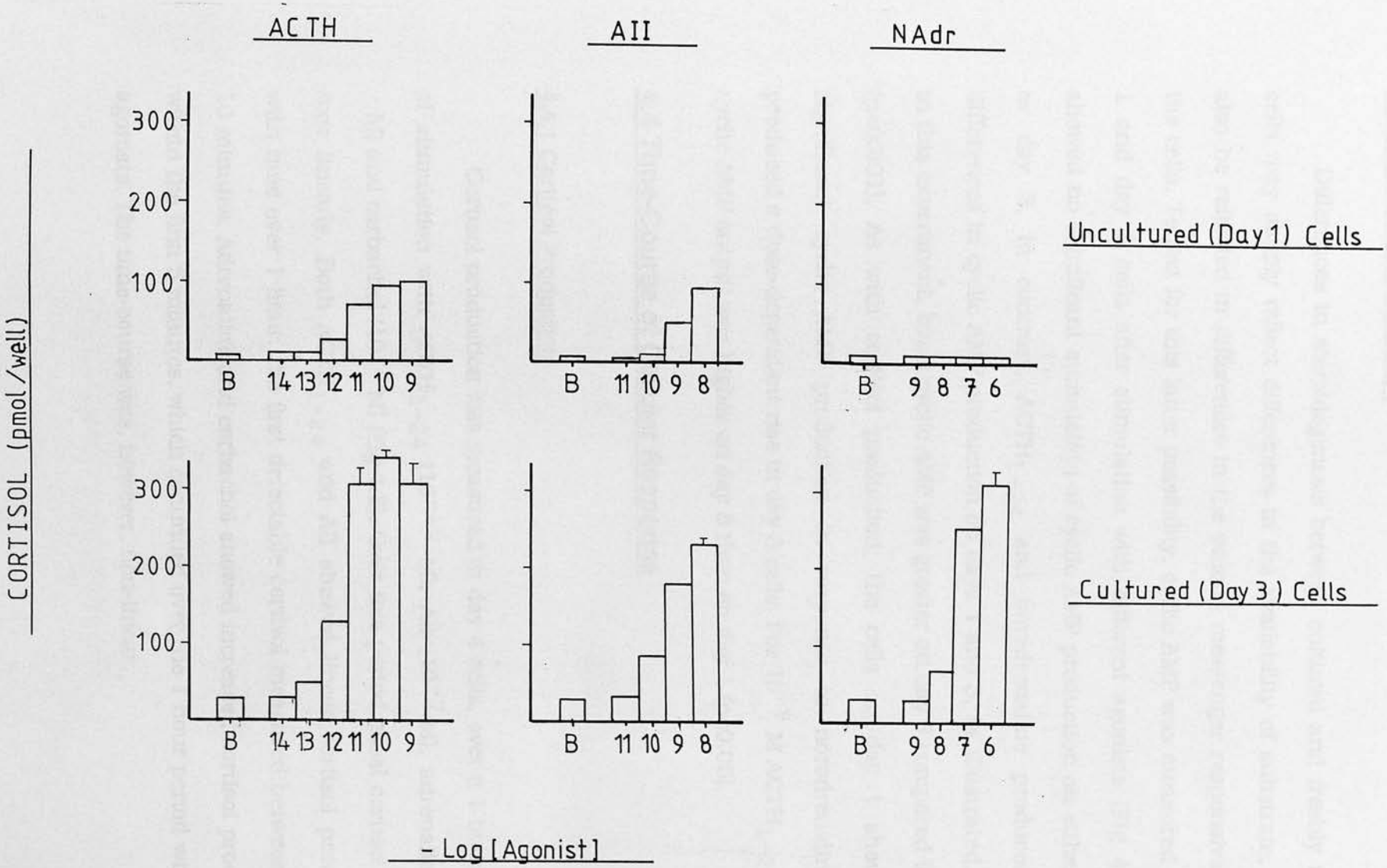


Fig 4.1 *Upper graphs* show cortisol produced by a 1 hour stimulation of freshly isolated (day 1) cells with increasing concentrations of the agonists stated. *Lower graphs* show cortisol produced by a 1 hour stimulation of day 3 cells with increasing concentrations of the agonists stated. B = basal. Representative results (1 of 3 experiments).

4.3.2 Cyclic AMP Production

Differences in steroidogenesis between cultured and freshly isolated cells may simply reflect differences in the availability of substrate. It may also be reflected in differences in the second messenger responsiveness of the cells. To test for this latter possibility, cyclic AMP was measured in day 1 and day 3 cells after stimulation with different agonists (Fig 4.2). AII showed no significant stimulation of cyclic AMP production on either day 1 or day 3. In contrast, $ACTH_{1-24}$ and noradrenaline produced clear differences in cyclic AMP production on days 1 and 3, as illustrated. Again, in this experiment, basal cyclic AMP was greater on day 3 compared to day 1 ($p<0.001$). As with cortisol production, the cells on day 1 showed no significant cyclic AMP production in response to noradrenaline, but produced a dose-dependant rise in day 3 cells. For 10^{-9} M $ACTH_{1-24}$, total cyclic AMP output was higher on day 3 than on day 1 ($p<0.05$).

4.4 Time-Course of Cellular Response

4.4.1 Cortisol Production

Cortisol production was measured in day 4 cells, over a 1 hour period of stimulation with $ACTH_{1-24}$ (10^{-10} M), AII (10^{-7} M), adrenaline (10^{-6} M) and carbachol (10^{-3} M) (Fig 4.3). Over this period basal cortisol output rose linearly. Both $ACTH_{1-24}$ and AII showed linear cortisol production with time over 1 hour, the first detectable cortisol measured between 5 and 10 minutes. Adrenaline and carbachol showed increased cortisol production within the first 5 minutes, which continued over the 1 hour period with both agonists. The time-course was, however, non-linear.

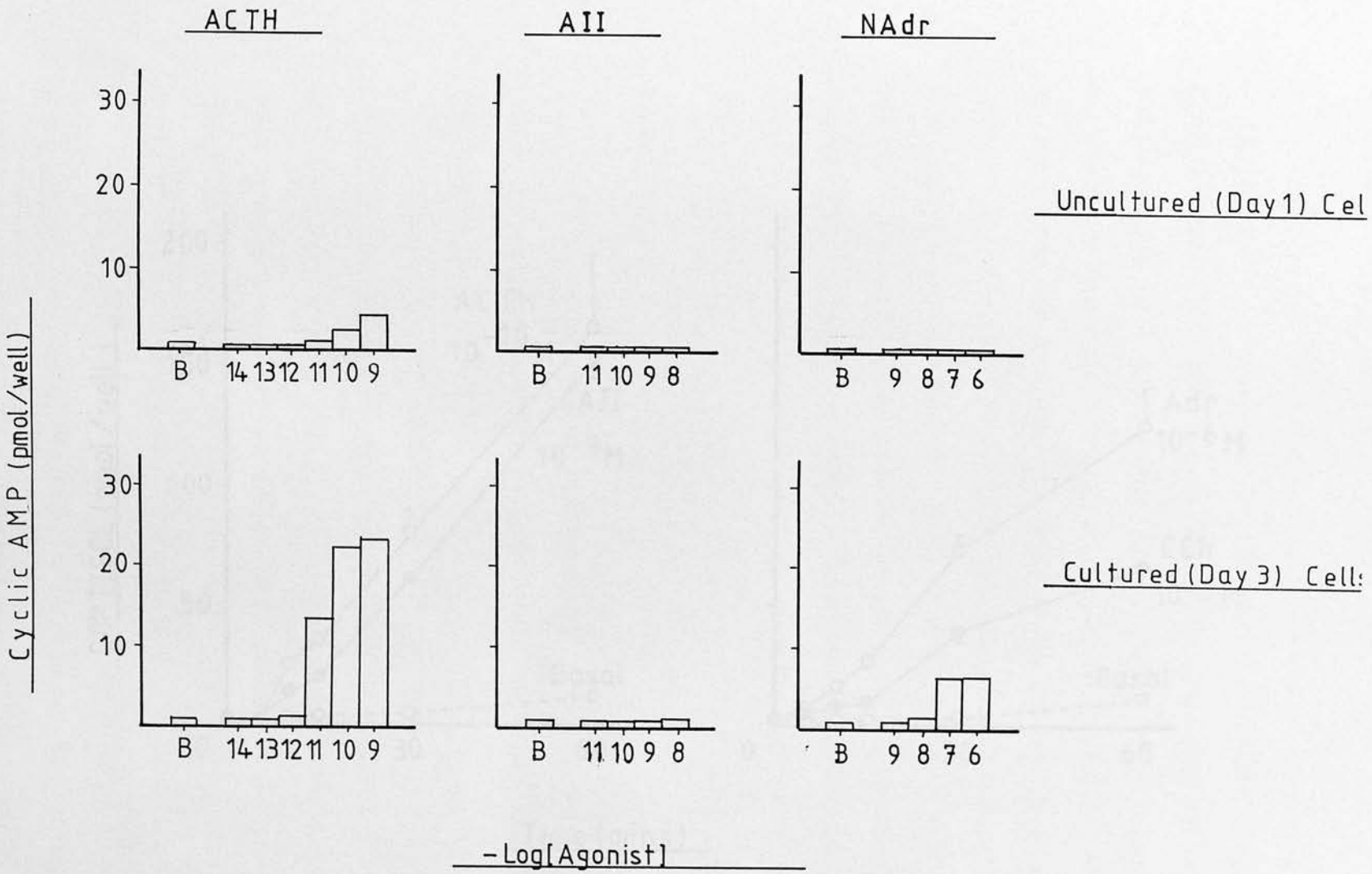


Fig 4.2 *Upper graphs* show combined (medium + cellular) cyclic AMP produced by a 1 hour stimulation of freshly isolated (day 1) cells with increasing concentrations of the agonists stated. *Lower graphs* show combined (medium + cellular) cyclic AMP produced by a 1 hour stimulation of day 3 cells with increasing concentrations of the agonists stated. B = basal. Results of a single experiment.

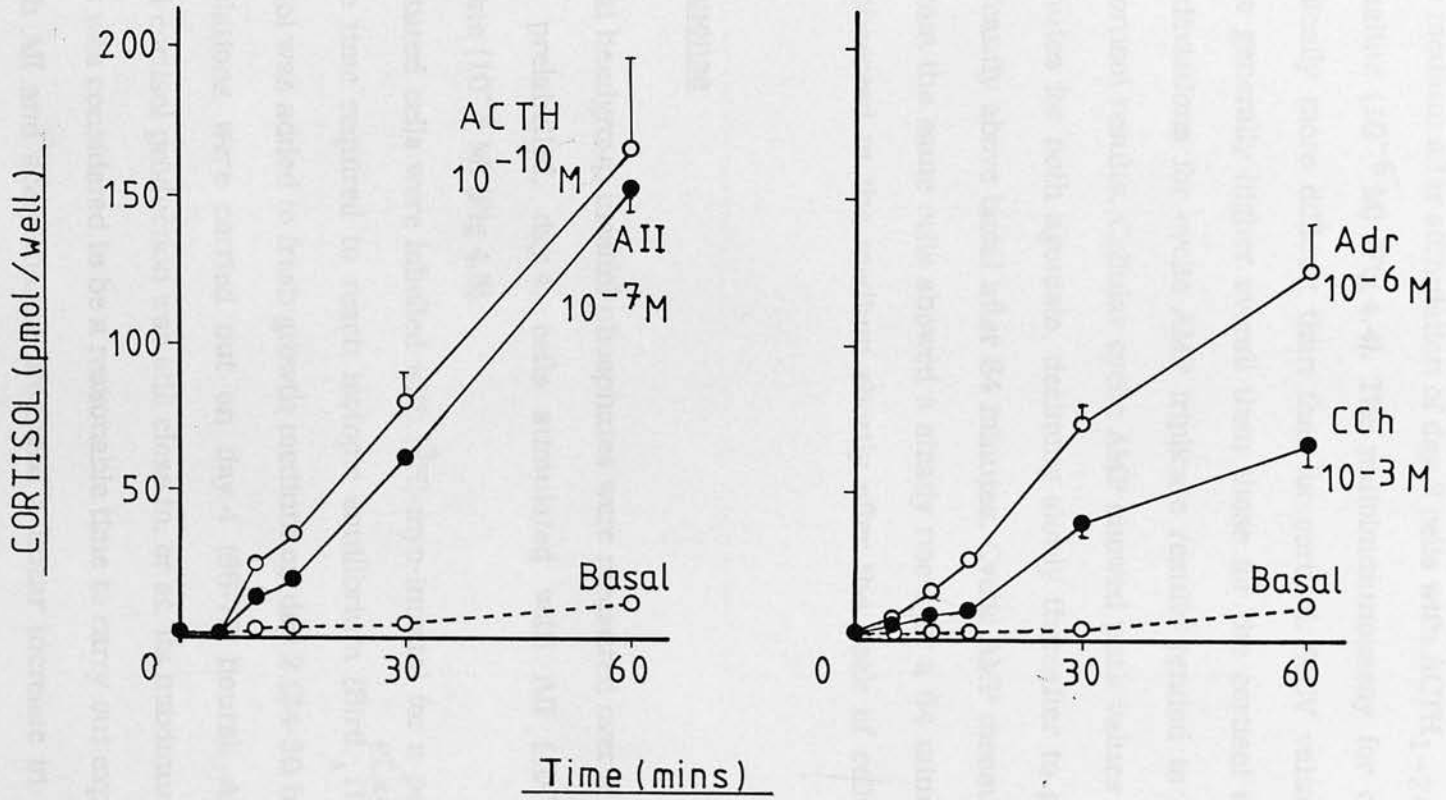


Fig 4.3 Time-course of cortisol production from day 3 cells upon stimulation with agonists stated. Representative results (1 of 3 experiments).

4.4.2 Cyclic AMP Production

Cyclic AMP production was measured in ethanol extracts of the cells and in the medium after stimulation of day 3 cells with $ACTH_{1-24}$ (10^{-9} M) and adrenaline (10^{-6} M) (Fig 4.4). The radioimmunoassay for cyclic AMP was technically more difficult than that for cortisol. %CV values for this assay were generally higher overall than those for the cortisol assay, and standard deviations for cyclic AMP triplicate results tended to be greater than for cortisol results. Cellular cyclic AMP showed peak values between 1 and 4 minutes for both agonists, declining slowly thereafter to give values still significantly above basal after 64 minutes. Cyclic AMP measured in the medium from the same cells showed a steady rise over a 64 minute period, and was observed in the medium shortly after the peak of cellular cyclic AMP.

4.4.3 PI response

Total headgroup inositol phosphates were measured over a 30 minute period in prelabelled, day 4, cells stimulated with AII (10^{-8} M) and acetylcholine (10^{-4} M) (Fig 4.5).

Cultured cells were labelled with [^{3}H]-myo-inositol for a period of 42 hours, the time required to reach isotopic equilibrium (Bird, *et al.* 1989). [^{3}H]-myo-inositol was added to fresh growth medium on day 2 (24-30 hours), and cell stimulations were carried out on day 4 (66-72 hours). As agonist-stimulated cortisol production was still close to, or at, its maximum value on day 4, this was considered to be a reasonable time to carry out experiments.

Both AII and acetylcholine produced a linear increase in total head group inositol phosphates with time for at least the first 15 minutes of stimulation. The results also showed that acetylcholine produced the first

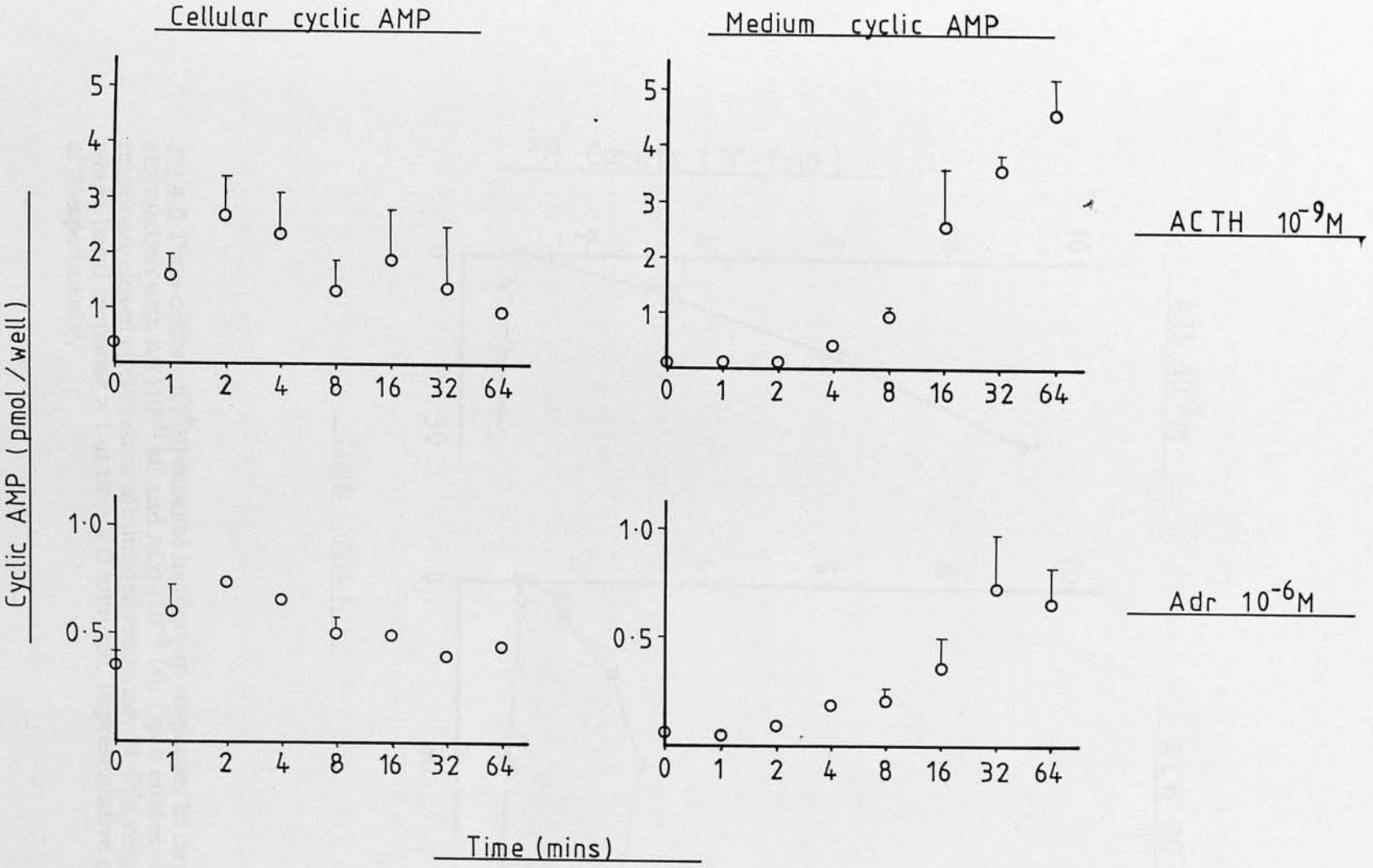


Fig 4.4 Time-course of cyclic AMP production, from day 3 cells, upon stimulation with $ACTH_{1-24}$ (10^{-9} M) or Adr (10^{-6} M), showing cellular cyclic AMP levels from extracted cells and cyclic AMP levels measured in the medium overlying the cells. Representative results (1 of 2 experiments).

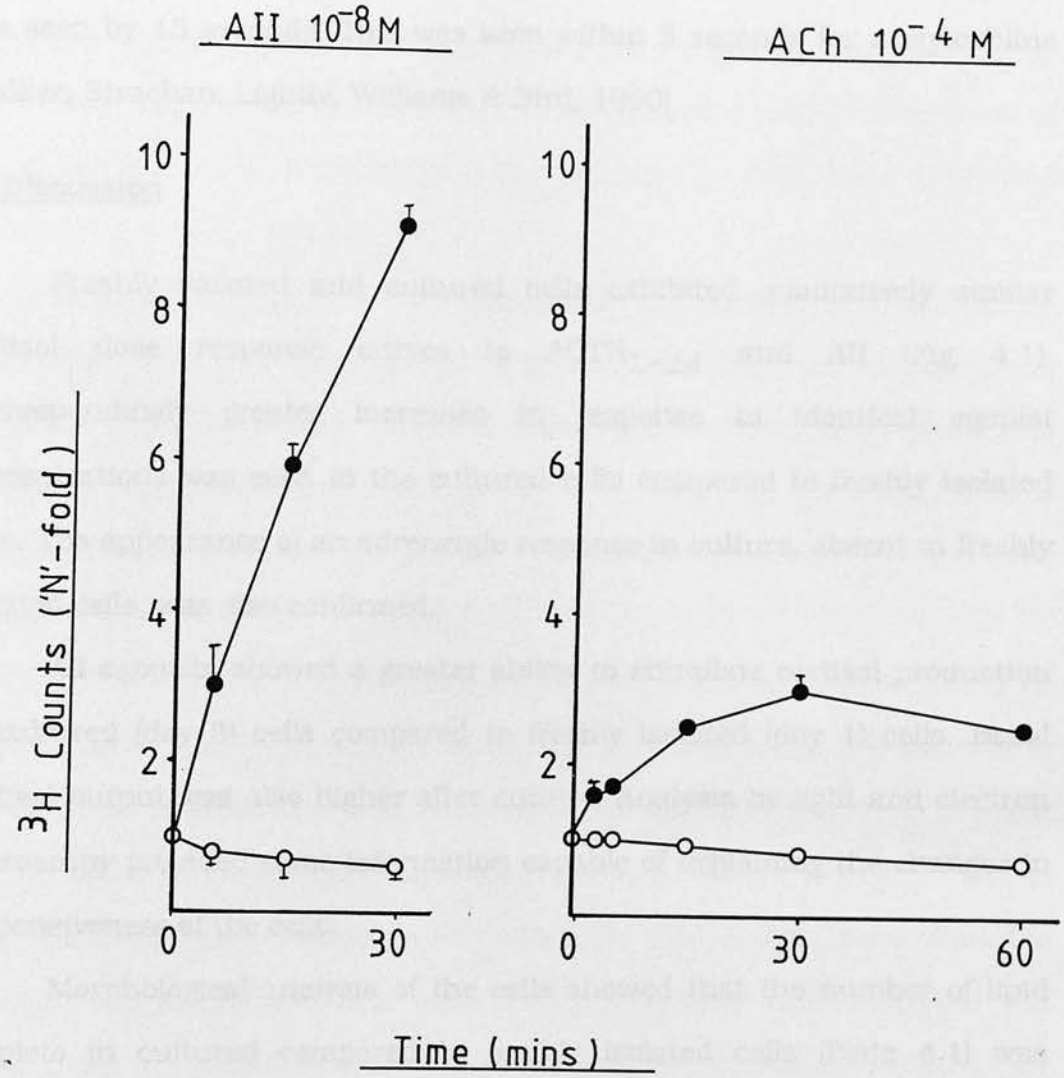


Fig 4.5 Time-course of [^{3}H]-inositol headgroup response, in day 4 cells, after stimulation with AII (10^{-8} M) and ACh (10^{-4} M). Open circles show basal response, closed circles show stimulated response. 'N'-fold response shown, where basal response = 1 at time = 0 minutes, Representative experiment (1 of 2 experiments).

measurable response by at least 2 minutes and AII by at least 5 minutes. More detailed analysis (Bird *et al*, 1989) showed that for AII, significant accumulation of the earliest product of phospholipase C action on phosphoinositide 4,5-bisphosphate (namely inositol (1,4,5) tris-phosphate) was seen by 15 seconds. This was seen within 5 seconds for acetylcholine (Walker, Strachan, Lightly, Williams & Bird, 1990).

4.5 Discussion

Freshly isolated and cultured cells exhibited qualitatively similar cortisol dose response curves to $ACTH_{1-24}$ and AII (Fig 4.1). Correspondingly greater increases in response to identical agonist concentrations was seen in the cultured cells compared to freshly isolated cells. The appearance of an adrenergic response in culture, absent in freshly isolated cells, was also confirmed.

All agonists showed a greater ability to stimulate cortisol production in cultured (day 3) cells compared to freshly isolated (day 1) cells. Basal cortisol output was also higher after culture. Analysis by light and electron microscopy provided some information capable of explaining the changes in responsiveness of the cells.

Morphological analysis of the cells showed that the number of lipid droplets in cultured compared to freshly isolated cells (Plate 4.1) was significantly greater. This may be reflected in a greater availability of steroidogenic substrate, providing one possible reason for an increased cortisol response. Cells in culture also showed more clearly defined plasma membranes, with improved ultrastructural integrity, which might also contribute to the difference. These ultrastructural differences might also underlie the improved second messenger responses, as evidenced by greater

agonist-stimulated cyclic AMP production of the cultured cells (Fig 4.2).

Recovery from both the stress-induced hormone changes in newly killed cattle and from collagenase digestion may also be contributory factors.

During the preliminary process of the characterisation of a primary culture system for bovine ZFR cells, it was shown that $ACTH_{1-24}$ failed to stimulate aldosterone production from these cells, confirming the absence of significant ZG cell contamination (Williams, Lightly, Ross, Bird & Walker, 1989).

Both $ACTH_{1-24}$ and AII maintained a constant rate of cortisol production over a 1 hour period (Fig 4.3), implying that steroidogenic substrate must be readily available within the cultured cells. The time-course of adrenaline-stimulated cortisol production was not linear. This is discussed later (section 5.5). The time-course of carbachol was also non-linear.

Cortisol appeared in the medium bathing the cells between 5 and 10 minutes after $ACTH_{1-24}$ and AII addition, and within the first 5 minutes of the addition of adrenergic and cholinergic agonists (Fig 4.3). It is possible that, if the adrenergic and cholinergic mechanisms are present *in vivo*, they could stimulate cortisol production from the adrenal faster than the hypothalimic-pituitary-adrenal axis. Using a system similar to the cell superfusion apparatus for freshly isolated cells, but in this case adapted for cultured cells, it should be possible to measure the time-course of cortisol production with the different agonists more accurately.

The time-course of intracellular cyclic AMP production upon stimulation with $ACTH_{1-24}$ and adrenaline (Fig 4.4) established that cyclic AMP levels had started to rise within the first minute of stimulation, reaching a peak between 1 and 4 minutes. In the case of cyclic AMP, *on*

the time scale measured, there was no difference in the timing of the responses to $ACTH_{1-24}$ and adrenaline. The cell superfusion system mentioned above would not be of any use in determining more accurately the timing of the cyclic AMP response, as it is the rise in intracellular cyclic AMP that is important. If supply of agonist to the cells could be adequately controlled, and the reaction could be terminated by some chemical or physical means (eg. liquid nitrogen) it may be possible to determine the time-course more accurately.

There appeared to be a mechanism for extrusion of cyclic AMP into the extracellular medium where cyclic AMP was observed after at least 4 minutes. *In vivo,* this may be a form of ~~intra~~*inter*cellular communication, possibly acting via gap-junctions.

The time-courses of total inositol phosphate production in response to AII (10^{-8} M) or acetylcholine (10^{-4} M) were linear for at least the first 15 minutes (Fig 4.5). Bird *et al* (1989) have shown that in these cells, a rise in inositol (1,4)bis-phosphate can be detected within the first 15 seconds upon stimulation with 10^{-7} M AII. They also concluded that inositol (1,4)bis-phosphate was formed by dephosphorylation of inositol (1,4,5)tris-phosphate, the most likely initial product of AII-stimulated phospholipase C activation. Walker, Strachan, Lightly, Williams & Bird (1990) have shown that, in these cells the formation of inositol(1,4,5)tris-phosphate occurs within 5 seconds of stimulation with acetylcholine (10^{-4} M) and precedes the production of all other inositol phosphates. Thus, AII and acetylcholine produce steroidogenesis in primary cultures of bovine adrenocortical zona fasciculata / reticularis via activation of phospholipase C.

Further study of the adrenergic and cholinergic responses within these cells required identification of suitable experimental conditions for the

measurement of steroid products and second messengers. Experiments involving purely cortisol measurement were carried out on day 3 or 4 cells after a 1 hour agonist stimulation. Where both cyclic AMP and cortisol measurements were required this was carried out on medium from agonist stimulated cells after 1 hour. Where a more accurate measurement of intracellular cyclic AMP was required, ethanol extraction of cells after 5 minutes agonist stimulation was used. For experiments involving phosphoinositide turnover, agonist stimulation was terminated after 15 minutes by the addition of perchloric acid (section 2.2.7.2), at which point the time-course of total inositol phosphates was still linear. In these experiments cells stimulated in parallel were used to assess cortisol output and combined (medium + cellular) cyclic AMP after a 15 minute period.

Hence, the results presented in this chapter (most of which have been published as: Williams, Lightly, Ross, Bird & Walker, 1988) show that day 3 cells gave qualitatively similar cortisol production in response to $ACTH_{1-24}$ & AII compared to day 1 cells, but show the appearance of a response to adrenergic agonists. Ultrastructural comparison of day 1 and 3 cells show improved integrity of day 3 cells, also reflected in increased cortisol output and cyclic AMP production of cells in culture. Studies of the second messengers produced upon agonist stimulation of cultured cells also gave information serving as a base for further studies of agonist responses.

5 The Adrenergic Response

5.1 Introduction

Results of stimulating cells with adrenergic and cholinergic agonists over 5 days (Fig 3.5), and of comparison between adrenergic dose responses of cells on day 1 and day 3 (Fig 4.1) showed that, though the cells failed to respond to catecholamines after initial isolation, after 12-18 hours (day 2 cells) of culture adrenergic stimulation produced steroidogenesis.

This chapter presents results of further study of the adrenergic response, concentrating on the nature of the second messengers involved, initial classification into alpha or beta response, and a study of desensitisation of the cells to adrenergic agonists.

5.2 Dose Responses to Catecholamines

Day 3 cells were stimulated with increasing doses of the catecholamines adrenaline (Adr), noradrenaline (NAdr) and isoprenaline (Isop). Cortisol was measured in the medium after 2 hours as already described, and results are shown in Fig 5.1. All 3 agonists showed similar dose response profiles. The threshold dose for each was 10^{-8} M ($p<0.05$) relative to basal and all agonists produced maximum cortisol output between 10^{-6} and 10^{-5} M.

Fig 5.2 shows corticosterone measured in the medium from the same experiment. Corticosterone levels were 15-30% of cortisol levels in the medium. Again dose responses were similar, though not identical for all three catecholamines. In this case the threshold dose was 10^{-7} M ($p<0.01$) for adrenaline and noradrenaline but 10^{-8} M ($p<0.05$) for isoprenaline.

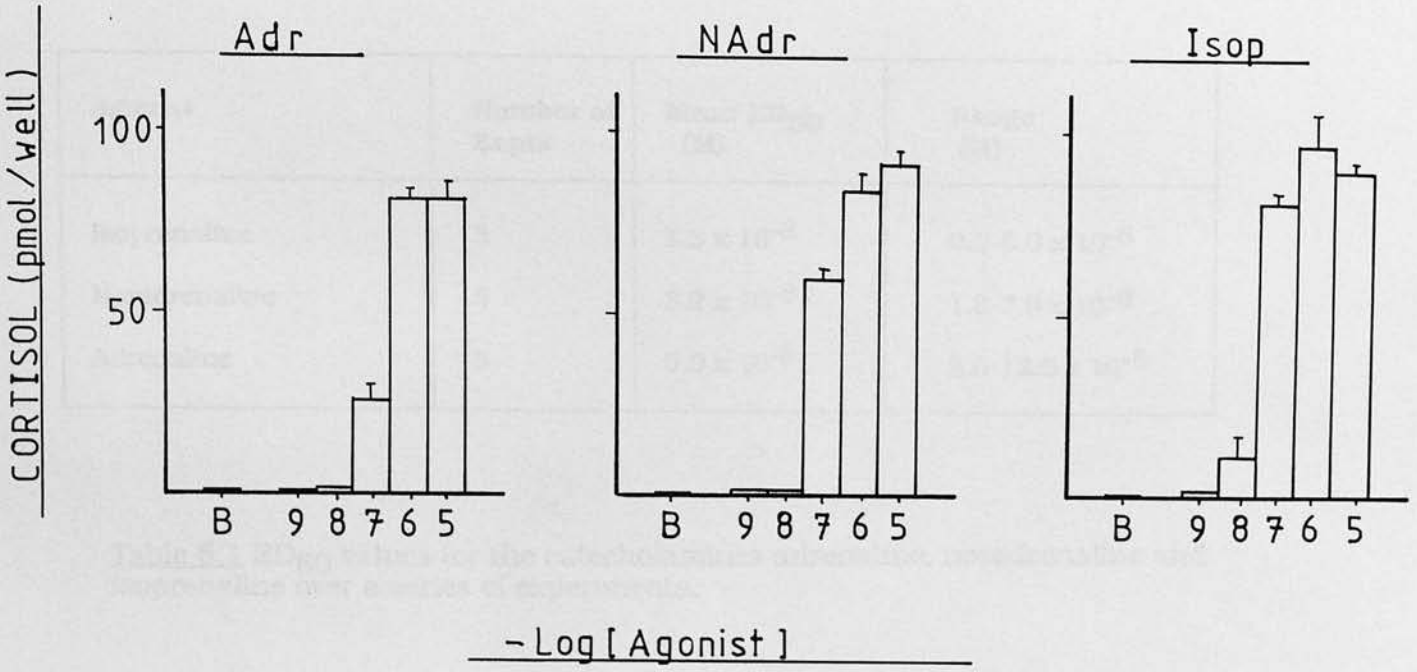


Fig 5.1 Cortisol produced by a 2 hour stimulation of day 3 cells with increasing concentrations of Adr, NAdr and Isop. B = basal. Representative experiment (1 of 3 experiments, except NAdr which was repeated 5 times).

Agonist	Number of Expts	Mean ED_{50} (M)	Range (M)
Isoprenaline	3	1.3×10^{-8}	$0.3\text{-}5.0 \times 10^{-8}$
Noradrenaline	5	3.2×10^{-8}	$1.3\text{-}7.9 \times 10^{-8}$
Adrenaline	3	5.0×10^{-8}	$2.0\text{-}12.6 \times 10^{-8}$

Table 5.1 ED_{50} values for the catecholamines adrenaline, noradrenaline and isoprenaline over a series of experiments.

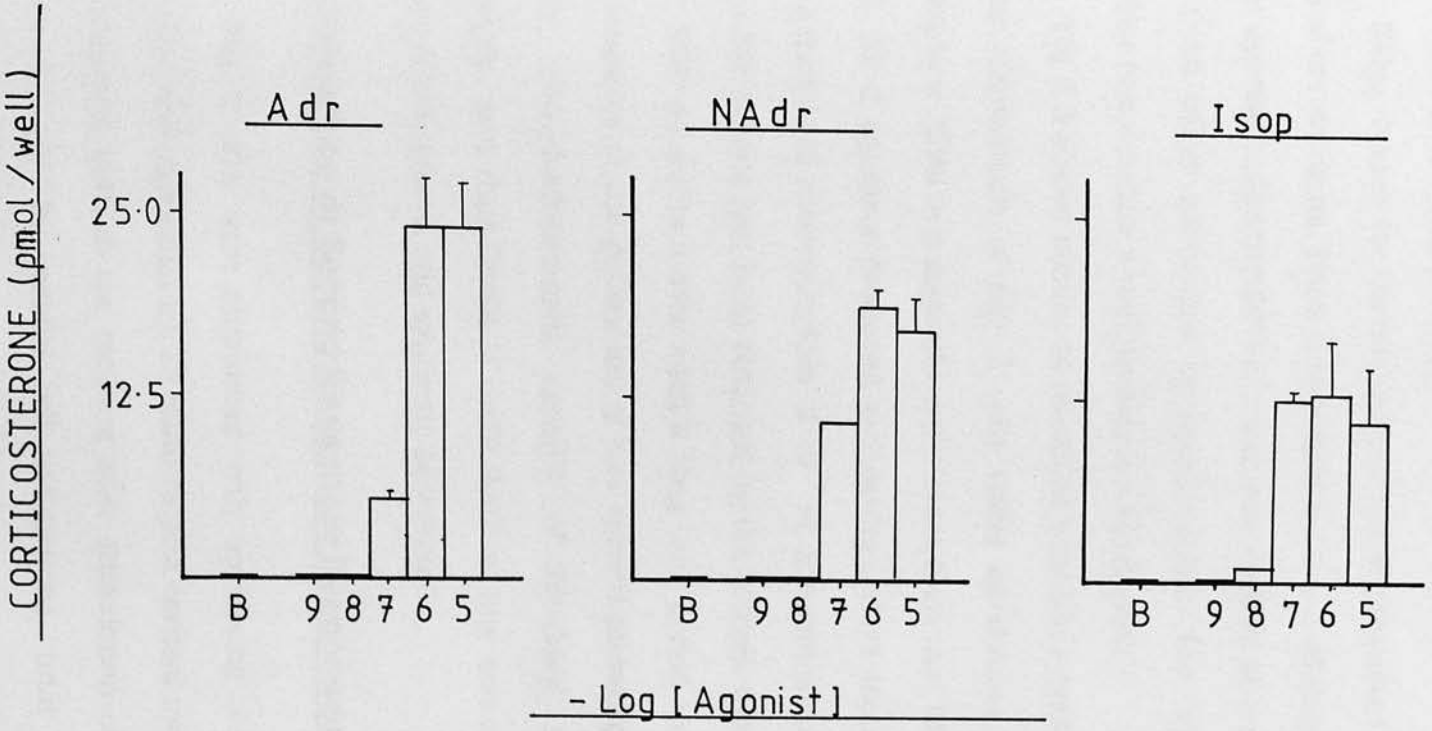


Fig 5.2 Corticosterone produced by a 2 hour stimulation of day 3 cells with increasing concentrations of Adr, NAdr and Isop. B = basal. Representative experiment (1 of 3 experiments; results from the same experiment as Fig 5.1).

Maximum cortisol output was achieved by 10^{-6} M for all agonists, but the maximum value decreased in the order adrenaline > noradrenaline > isoprenaline in this experiment.*

ED_{50} values for cortisol output are summarised in table 5.1 over a series of experiments. From this table it is clear that isoprenaline is the most potent agonist achieving 50% of maximum cortisol output (ED_{50}) at a lower dose than either adrenaline or noradrenaline. The order of potency was therefore isoprenaline > noradrenaline > adrenaline.

Fig 5.3 shows combined (medium + cellular) cyclic AMP levels after a 1 hour stimulation of day 3 cells using adrenaline, noradrenaline and isoprenaline. This is a separate experiment from that illustrated in Figs 5.1 & 5.2. All 3 agonists produced a dose-dependant increase in cyclic AMP, having threshold concentrations of 10^{-7} M. It is not clear whether maximum cyclic AMP output had been obtained by the highest doses of agonists used. Cyclic AMP production over such a long time period was not necessarily a valid measure of the generation of this second messenger because all cells contain phosphodiesterases capable of breaking down this second messenger, and thus levels of cyclic AMP at any one instant are a subtle balance of breakdown and synthetic processes.

5.3 Comparison of Second Messenger Responses

Day 4 cells were stimulated with increasing concentrations of AII, adrenaline and carbachol for 15 minutes and cortisol measured. Cyclic AMP was measured both in the medium after stimulation of these cells and in cellular extracts. In parallel cell incubations, total inositol phosphate headgroup production was measured according to the method described in section 2.2.7. Results are presented in Figs 5.4, 5.5 & 5.6.

* Over the series of experiments presented in table 5.1, observation indicated that adrenaline, noradrenaline and isoprenaline produced the same maximum cortisol production.

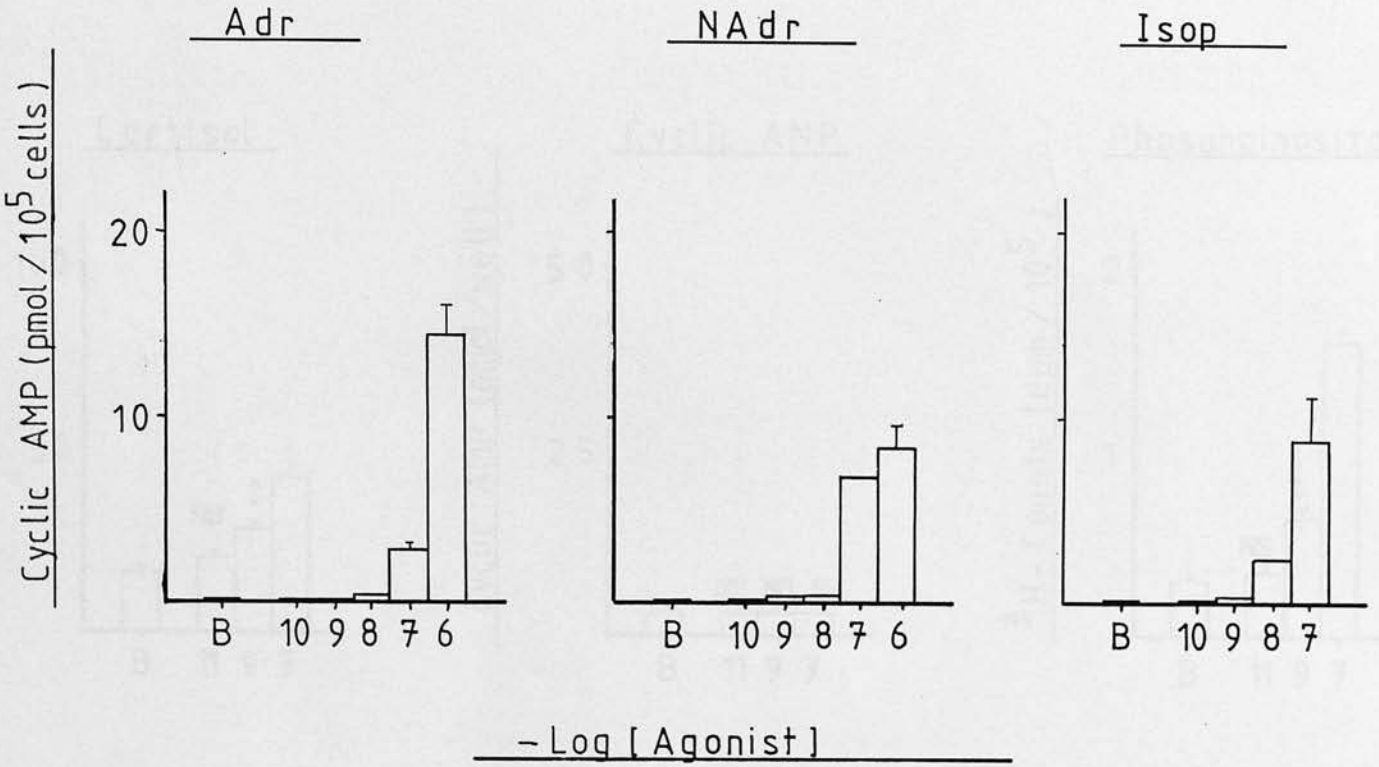


Fig 5.3 Combined (medium + cellular) cyclic AMP produced by a 1 hour stimulation of day 3 cells with increasing concentrations of Adr, NAdr and Isop. B = basal. Representative experiment (1 of 3 experiments).

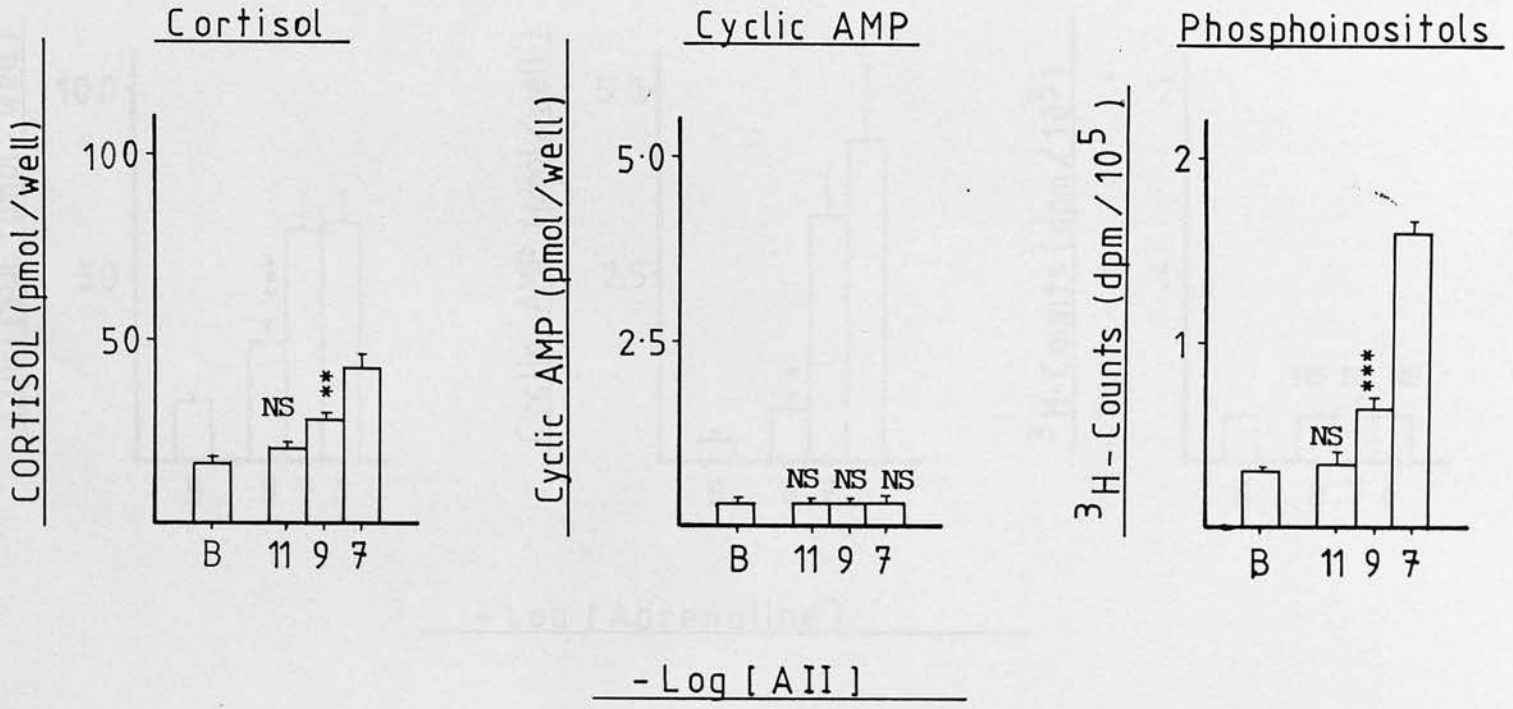


Fig 5.4 Cortisol, combined (medium + cellular) cyclic AMP and phosphoinositols produced over a 15 minute time period in day 4 cells upon stimulation with increasing concentrations of AII. Abbreviations are: NS, not significant; **, $p<0.01$; ***, $p<0.001$, $n = 3$. B = basal. Representative experiment (1 of 3 experiments).

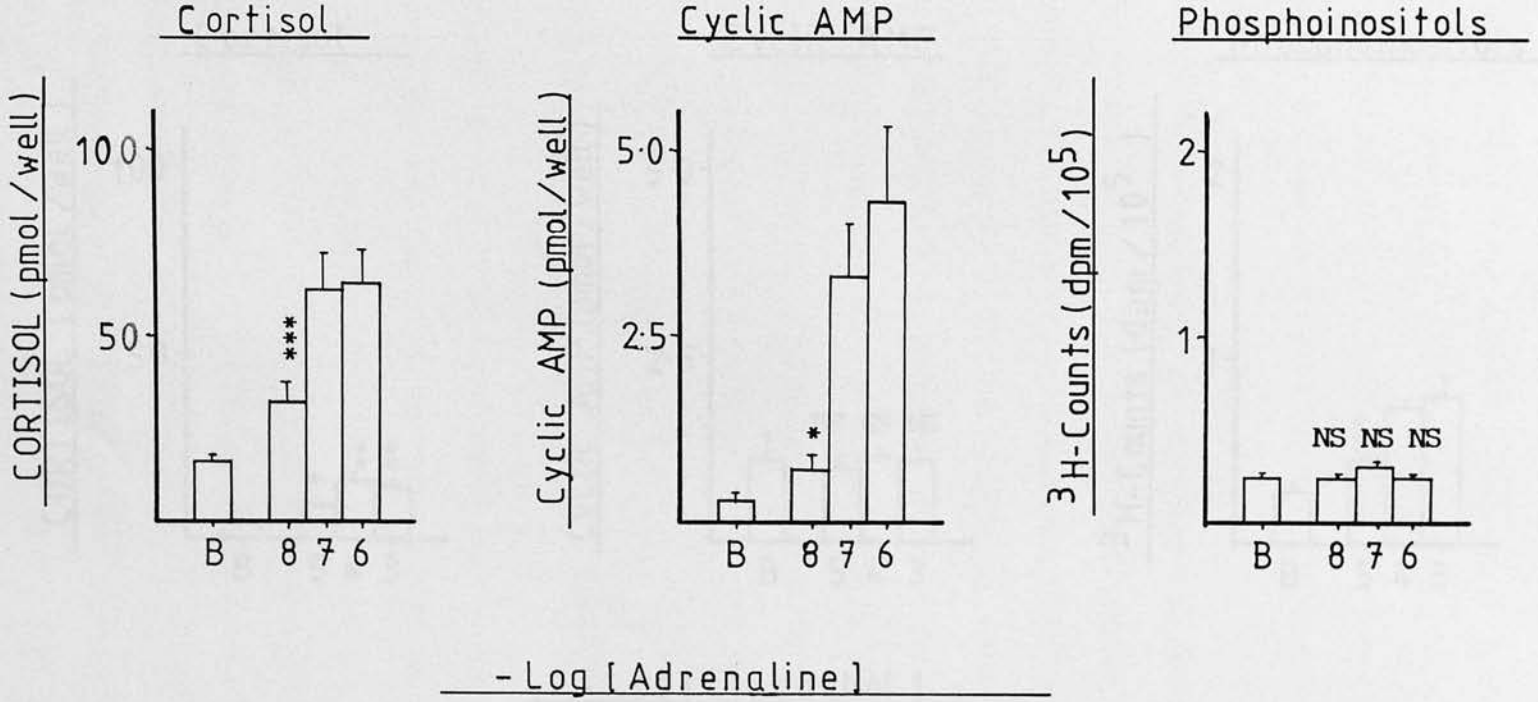


Fig 5.5 Cortisol, combined (medium + cellular) cyclic AMP and phosphoinositols produced over a 15 minute time period in day 4 cells upon stimulation with increasing concentrations of adrenaline. Abbreviations are: NS, not significant; *, $p<0.05$; ***, $p<0.001$; n = 3. B = basal. Representative experiment (1 of 3 experiments; results from the same experiment as Fig 5.4).

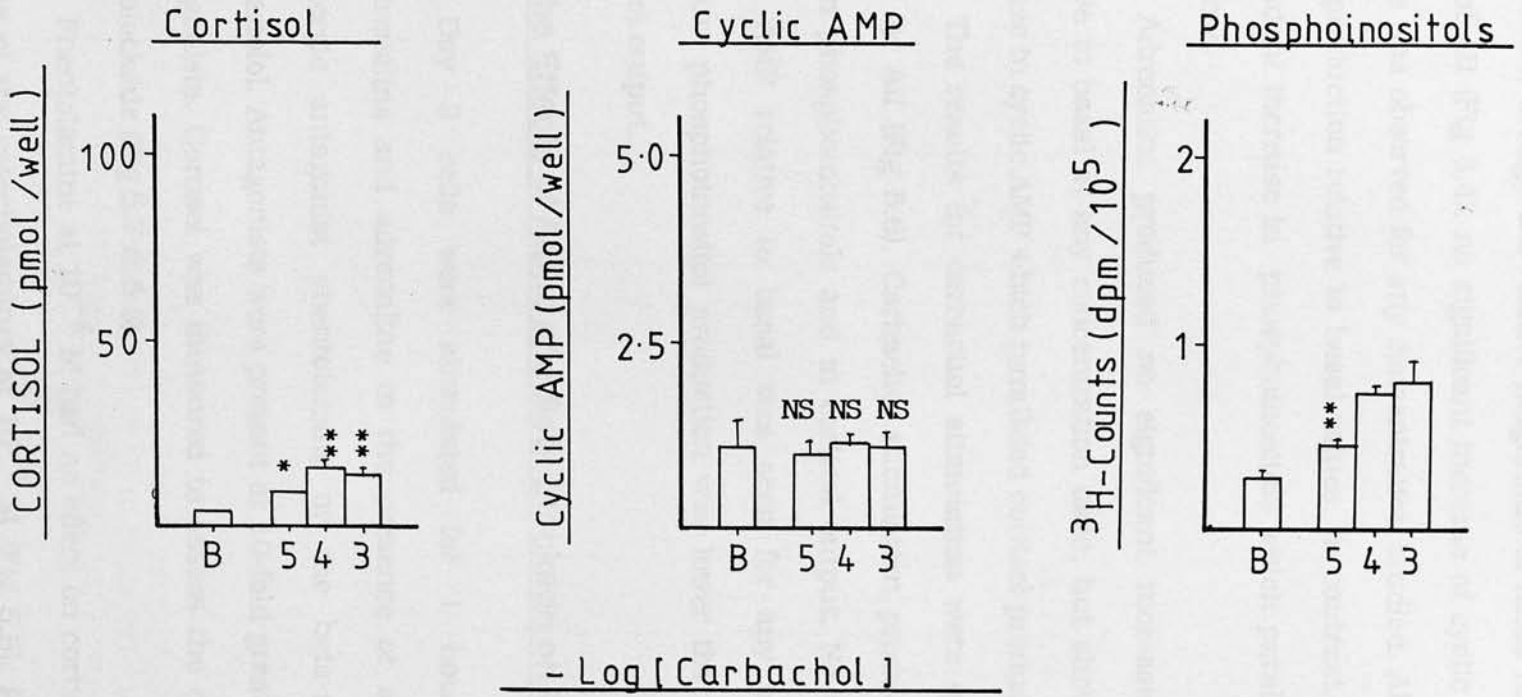


Fig 5.6 Cortisol, combined (medium + cellular) cyclic AMP and phosphoinositols produced over a 15 minute time period in day 4 cells upon stimulation with increasing concentrations of carbachol. Abbreviations are: NS, not significant; *, $p<0.05$; **, $p<0.01$; n = 3. B = basal. Representative experiment (1 of 3 experiments; results from the same experiment as Fig 5.4).

All 3 agonists produced dose-dependant increases in cortisol production, though the relative magnitude of these responses varied. In the case of AII (Fig 5.4), no significant increase of cyclic AMP relative to basal values was observed for any concentration studied. AII did not inhibit cyclic AMP production relative to basal values. In contrast, AII produced a dose-dependant increase in phosphoinositols which paralleled the cortisol dose response.

Adrenaline produced no significant increase in phosphoinositols relative to basal at any concentration used, but showed a dose-dependant increase in cyclic AMP which paralleled cortisol production (Fig 5.5).

The results for carbachol stimulation were qualitatively similar to those for AII (Fig 5.6). Carbachol stimulation produced a dose-dependant rise in phosphoinositols and in cortisol output. No significant increase in cyclic AMP relative to basal was seen for any concentration studied. Absolute phosphoinositol production was lower than that for AII,* as was cortisol output.

5.4 The Effects of Alpha and Beta Blockade on Cortisol Production

Day 3 cells were stimulated for 1 hour with isoprenaline, noradrenaline and adrenaline in the presence or absence of the alpha-adrenergic antagonist phentolamine or the beta-adrenergic antagonist propranolol. Antagonists were present at 10-fold greater concentration than the agonists. Cortisol was measured to assess the effects of the alpha or beta-blockade (Fig 5.7 & 5.8).

Phentolamine at 10^{-6} M had no effect on cortisol secretion produced by any of the catecholamines at 10^{-7} M (Fig 5.5). Propranolol at 10^{-6} M produced a 97% inhibition (stimulated relative to basal value) in cortisol

* for a maximum steroidogenic dose of AII (10^{-8} M) or carbachol (10^{-3} M)

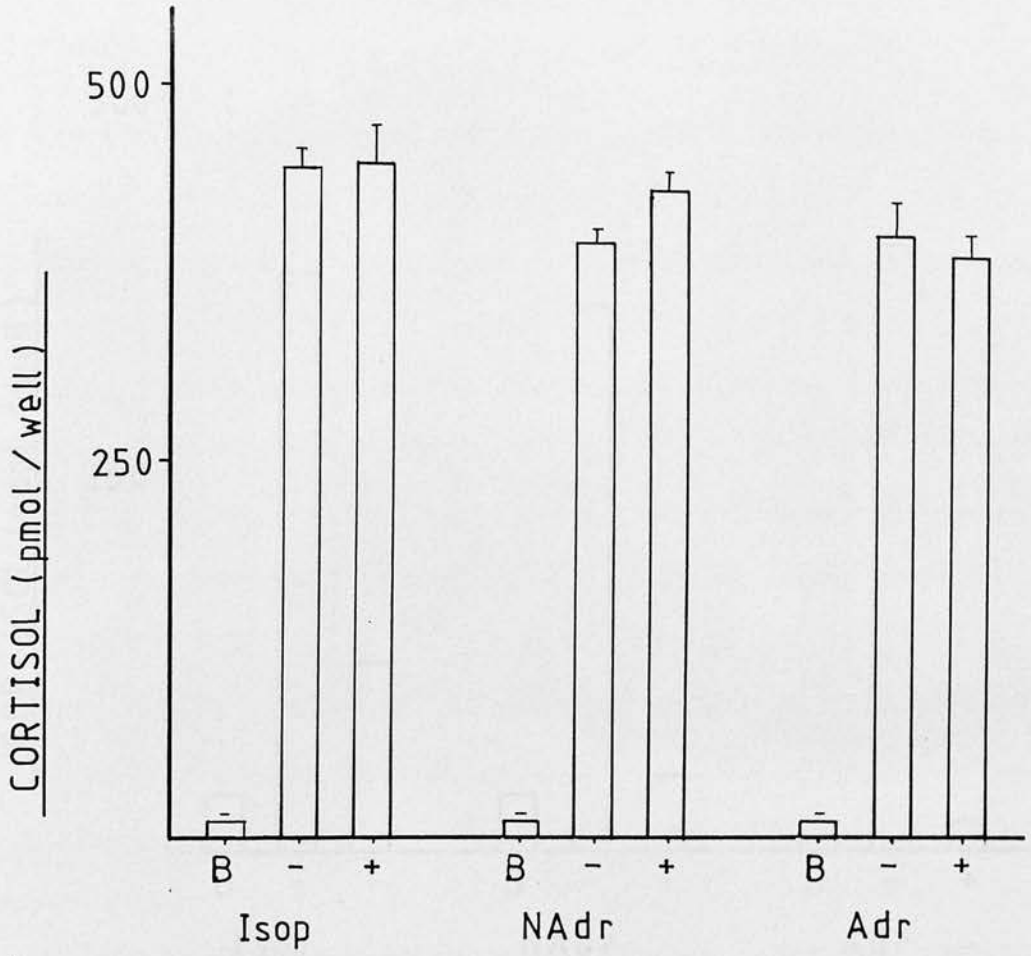


Fig 5.7 Cortisol produced by a 1 hour stimulation of day 3 cells with 10^{-7} M catecholamine in the presence (+) or absence (-) of 10^{-6} M phentolamine. B = basal. Representative experiment (1 of 3 experiments).

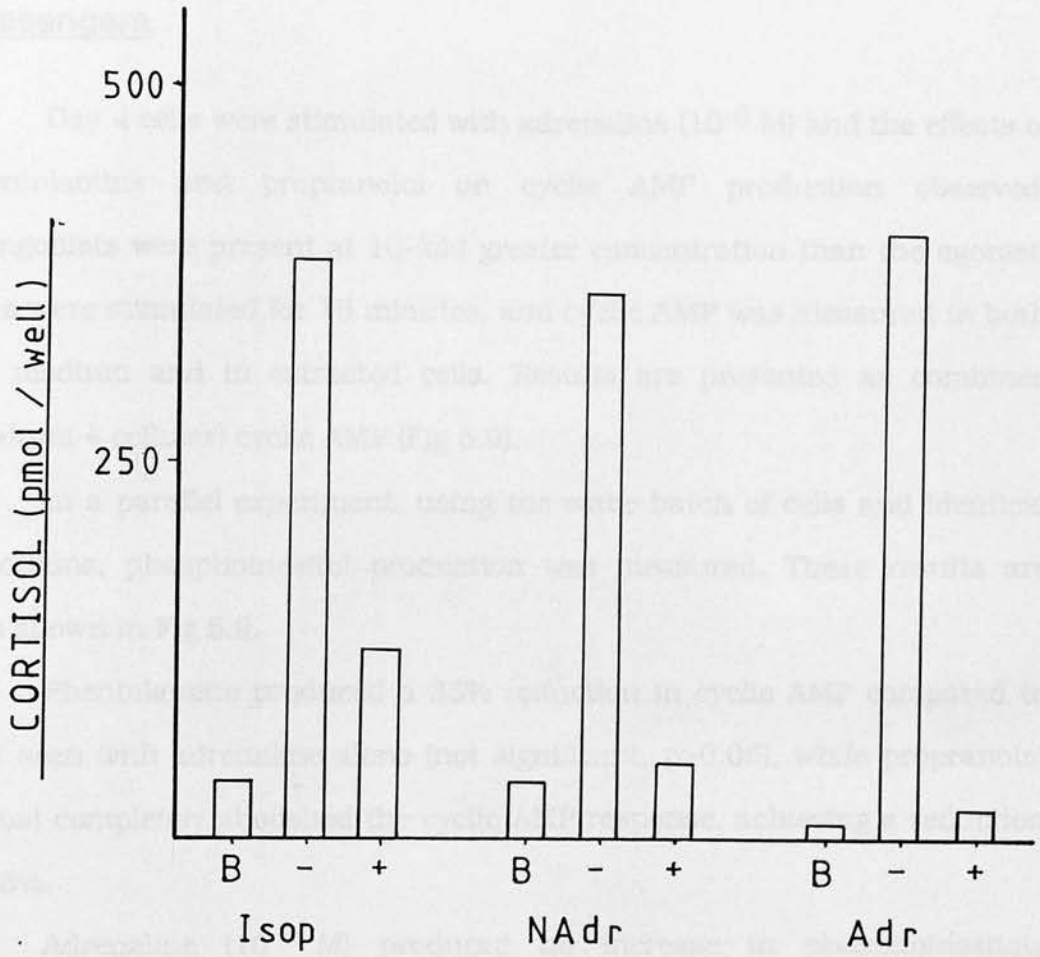


Fig 5.8 Cortisol produced by a 1 hour stimulation of day 3 cells with 10^{-7} M catecholamine in the presence (+) or absence (-) of 10^{-6} M propranolol. B = basal. Representative experiment (1 of 3 experiments).

production by 10^{-7} M adrenaline, and a 96% inhibition in cortisol production by 10^{-7} M noradrenaline. Propranolol inhibited cortisol produced by 10^{-7} M isoprenaline by 75%. Propranolol and phentolamine had no effect on basal cortisol production.

5.5 The Effect of Alpha and Beta Blockade on Cellular Second Messengers

Day 4 cells were stimulated with adrenaline (10^{-6} M) and the effects of phentolamine and propranolol on cyclic AMP production observed. Antagonists were present at 10-fold greater concentration than the agonist. Cells were stimulated for 15 minutes, and cyclic AMP was measured in both the medium and in extracted cells. Results are presented as combined (medium + cellular) cyclic AMP (Fig 5.9).

In a parallel experiment, using the same batch of cells and identical conditions, phosphoinositol production was measured. These results are also shown in Fig 5.9.

Phentolamine produced a 35% reduction in cyclic AMP compared to that seen with adrenaline alone (not significant, $p>0.05$), while propranolol almost completely abolished the cyclic AMP response, achieving a reduction of 98%.

Adrenaline (10^{-6} M) produced no increase in phosphoinositols relative to basal, and neither the basal nor the stimulated cell phosphoinositol production were affected by phentolamine or propranolol.

5.6 Effect of Growing Cells in Catecholamine Supplemented Medium

Cells prepared in the usual manner (section 2.2.2) were grown in

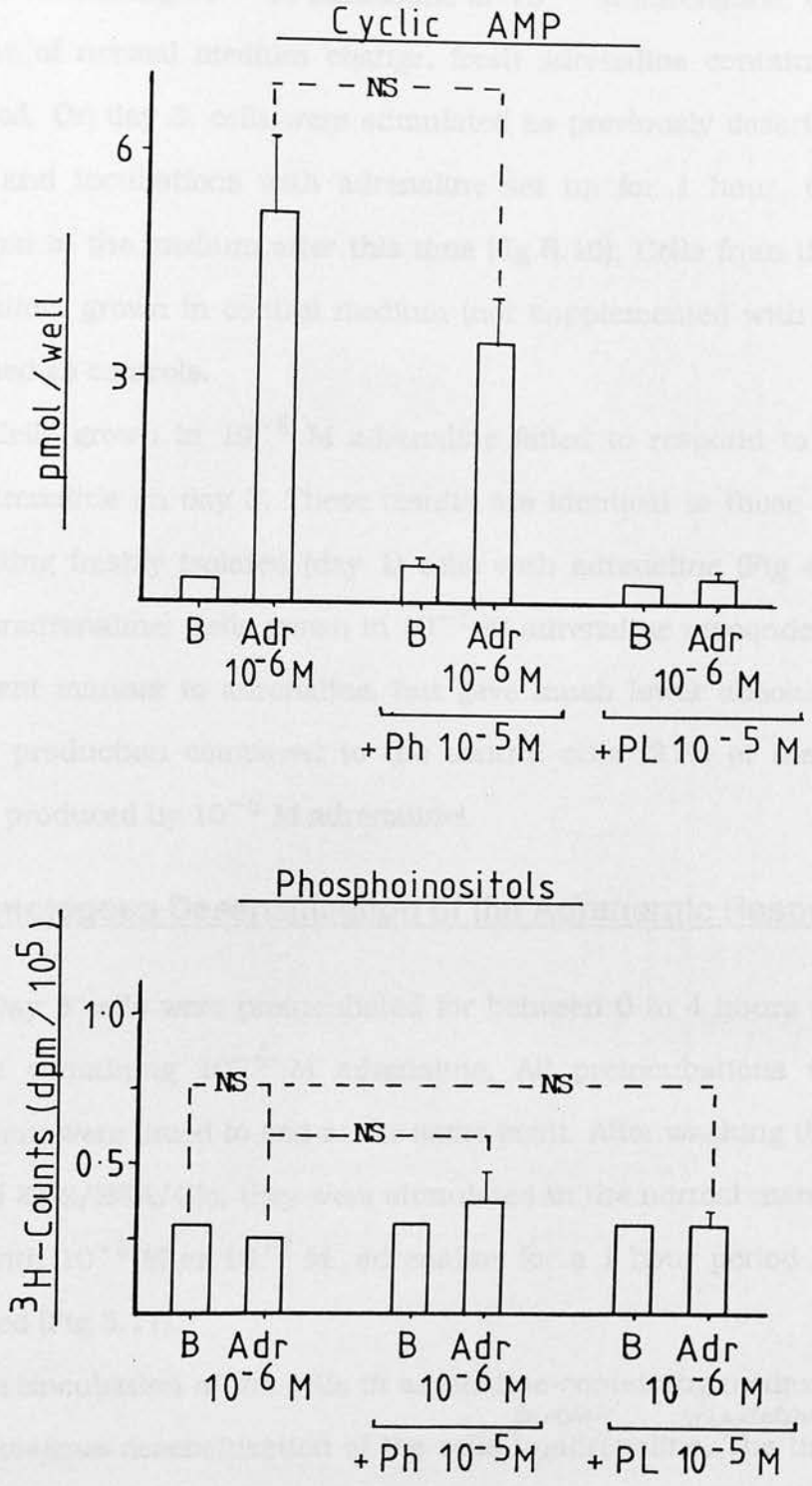


Fig 5.9 Combined (medium + cellular) cyclic AMP (top) and phosphoinositols (bottom) production over a 15 minute period in day 4 cells stimulated with adrenaline (10^{-6} M) in the presence or absence of phentolamine (Ph, 10^{-5} M) or propranolol (PL, 10^{-5} M). Abbreviation: NS, not significant. B = basal. Representative experiment (1 of 2 experiments).

medium containing 10^{-7} M adrenaline or 10^{-6} M adrenaline. On day 2, at the time of normal medium change, fresh adrenaline containing medium was used. On day 3, cells were stimulated as previously described (Section 2.2.3), and incubations with adrenaline set up for 1 hour. Cortisol was measured in the medium after this time (fig 5.10). Cells from the same cell preparation, grown in control medium (not supplemented with adrenaline), were used as controls.

Cells grown in 10^{-6} M adrenaline failed to respond to stimulation with adrenaline on day 3. These results are identical to those obtained by stimulating freshly isolated (day 1) cells with adrenaline (Fig 4.1 - in this case noradrenaline) Cells grown in 10^{-7} M adrenaline responded in a dose-dependent manner to adrenaline, but gave much lower absolute values of cortisol production compared to the control cells (21% of the amount of cortisol produced by 10^{-6} M adrenaline).

5.7 ~~Homologous~~ Desensitisation of the Adrenergic Response

Day 3 cells were preincubated for between 0 to 4 hours with growth medium containing 10^{-6} M adrenaline. All preincubations within each experiment were timed to end at the same point. After washing the cells with (3x) 1ml EBS/BSA/Glc, they were stimulated in the normal manner (section 2.2.3) with 10^{-6} M or 10^{-7} M adrenaline for a 1 hour period and cortisol measured (Fig 5.11).

Preincubation of the cells in adrenaline-containing medium produced ~~an homologous~~ desensitisation of the cells to further stimulation adrenaline. The time required to fall to 50% of the maximum response, produced by control cells with the same dose of agonist, varied between 30 minutes and 2 hours.

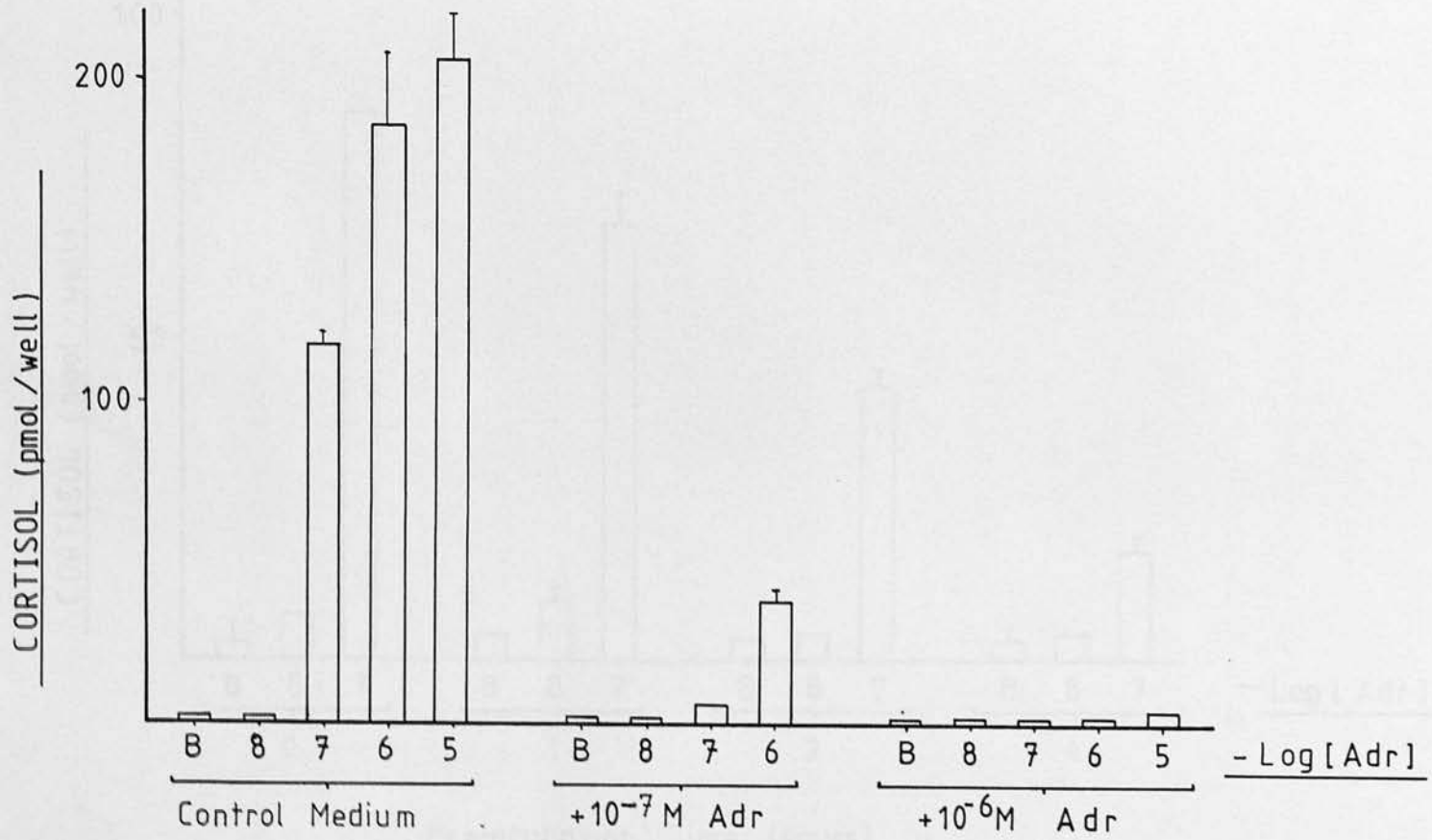


Fig 5.10 Cortisol produced by a 1 hour stimulation of day 3 cells with increasing concentrations of adrenaline. Cells grown, from time of plating, in control growth medium, medium containing 10^{-7} M adrenaline or medium containing 10^{-6} M adrenaline. B = basal. Representative experiment (1 of 2 experiments).

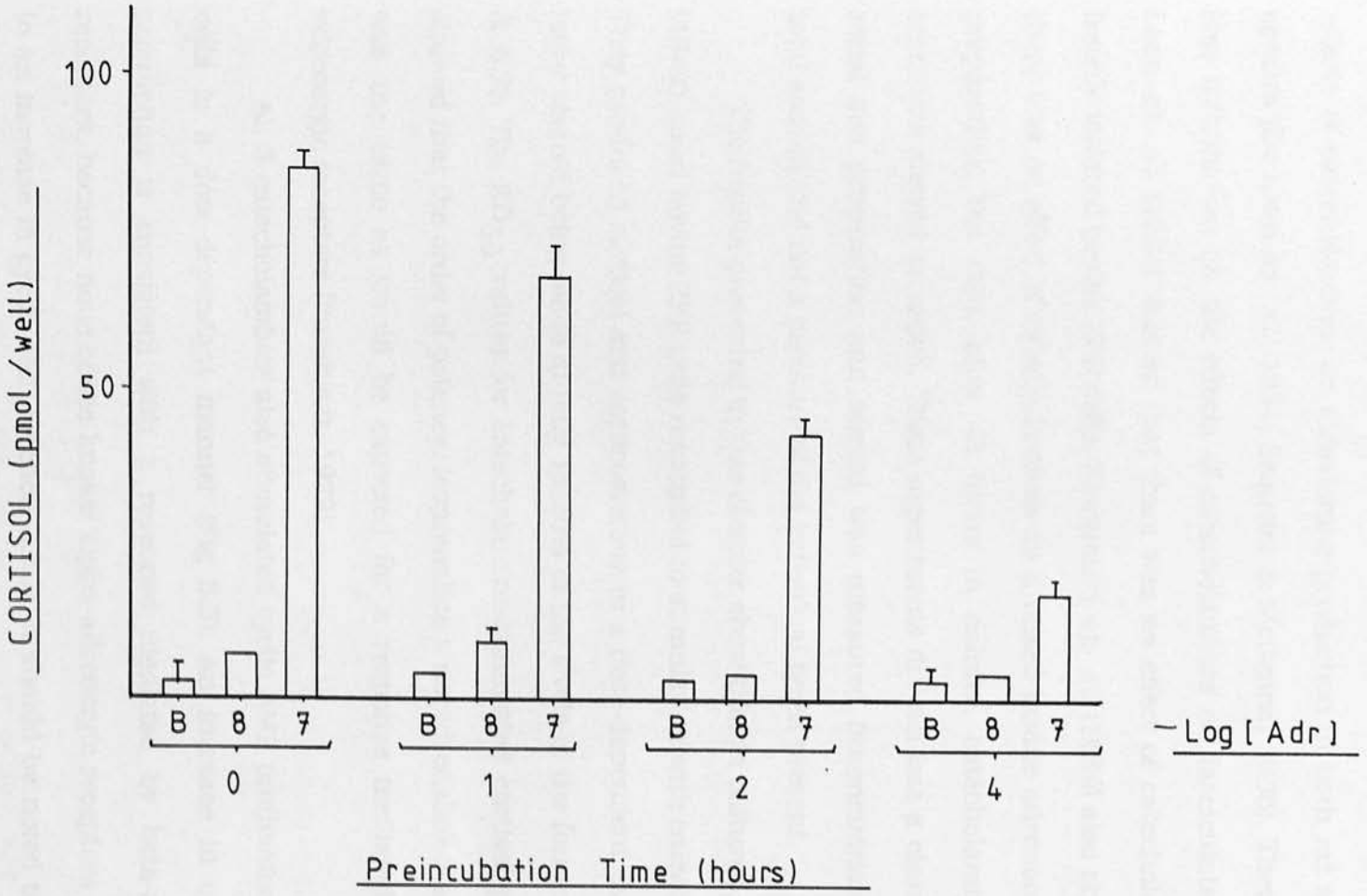


Fig 5.11 Cortisol produced by a 1 hour stimulation, with increasing concentrations of adrenaline, of day 3 cells which had been preincubated for varying times (0 - 4 hours) in growth medium supplemented with 10^{-6} M adrenaline. B = basal. Representative experiment (1 of 4 experiments).

5.8 Discussion

Previous workers in the field of adrenal research have reported the effects of catecholamines on aldosterone production in both rat and bovine species (De Lean *et al*, 1984; Sequeira & McKenna, 1985). There is much less information on the effects of catecholamines on fasciculata cells. De Lean *et al* (1984) showed that there was no effect of catecholamines on freshly isolated bovine ZFR cells. Kawamura *et al* (1984) also showed that there was no effect of catecholamines on a mixed bovine adrenocortical cell preparation, but that, after 48 hours in culture, catecholamines could stimulate steroid secretion. These experiments did not use a clearly defined zonal cell preparation and steroid was measured fluorometrically, giving total steroid and not a measure of the individual types present.

The results presented in this chapter show that in cultured day 3 or 4 (48-60 hour) bovine ZFR cells responded to stimulation with catecholamines. They produced cortisol and corticosterone in a dose-dependent manner, the latter steroid being made at only 15-30% of the levels of the former (Figs 5.1 & 5.2). The ED_{50} values for catecholamine-stimulated cortisol production showed that the order of potency, isoprenaline > noradrenaline > adrenaline, was the same as would be expected for a response mediated by beta-adrenergic receptors (Furchgott, 1972).

All 3 catecholamines also stimulated cyclic AMP production from the cells in a dose-dependant manner (Fig 5.3). An increase in cyclic AMP production is consistent with a response mediated by beta-adrenergic receptors, because none of the known alpha-adrenergic receptors are linked to an increase in cyclic AMP (Section 1.2.2). It should be noted that alpha-adrenoceptors could also be present, but not involved in steroidogenesis.

The evidence from studies of the second messengers in the cells (Figs

5.4, 5.5 & 5.6) also confirmed the involvement of cyclic AMP in stimulation of cortisol production by adrenergic agonists. This also showed that adrenergic agonists failed to stimulate phosphoinositol production in the cells. This was in contrast to the effects of AII which did not produce an increase in cellular cyclic AMP, relative to basal values, but gave a dose-dependant rise in phosphoinositols. Carbachol acted in a similar manner to AII in that it too appeared to mediate its steroidogenic effects via the phoshphoinositde-derived second messenger system. Thus it appears that the catecholamines produce steroidogenesis in cultured bovine ZFR cells via a cyclic AMP dependant mechanism and that AII and cholinergic agonists produce there effects via phosphoinositide breakdown.

Experiments with the specific alpha-antagonist phentolamine, and the specific beta-adrenergic antagonist propranolol (Fig 5.7 & 5.8), confirmed that beta-adrenoceptors were responsible for mediating the steroidogenic effect of these agonists on the cells.* A 10-fold excess of propranolol relative to adrenaline, noradrenaline or isoprenaline almost completely blocked cortisol production stimulated by each of these catecholamines. In identical experiments, phentolamine had no effect on catecholamine-stimulated cortisol production. Additionally, propranolol completely blocked adrenaline stimulated cyclic AMP production, while phentolamine appeared to reduce cyclic AMP production, but not to a significant degree. Phosphoinositol production was unaffected by either of the antagonists.

One of the arguments for the absence of an adrenergic response in freshly isolated (day 1) cells is that high levels of catecholamines in the adrenal caused by stress before the death of the animal may lead to a desensitisation of this response (Section 1.4.2). Growing cells in medium

* control exepriments showed that phentolamine and propranolol had no effect on cortisol secretion produced by $ACTH_{1-24}$ or AII.

supplemented with adrenaline (Fig 5.10) dramatically reduced the adrenergic response. Cells grown in medium containing 10^{-6} M adrenaline failed to produce cortisol in response to stimulation with adrenaline on day 3. (It should be noted that no account was taken of breakdown of catecholamine, as the medium was only changed every 24 hours, yet the cells were still subject to desensitisation of the adrenergic response). The absence of a dose-response to adrenaline is a similar situation to that seen with adrenergic stimulation of freshly isolated cells (see Fig 4.1). Growing the cells in catecholamine-supplemented medium may be simulating the local environment that the adrenocortical cells are present in shortly after the death of the animal.

The adrenal cells are unlikely to be subject to significant levels of catecholamines *in vivo*, unless there is an, as yet unproven, retrograde flow of blood between medulla and cortex. However, desensitisation of the adrenergic response of the adrenal cortex *in vivo* could also occur by prolonged stimulation via adrenergic innervation.

If the adrenergic response of the adrenocortical cells had been desensitised in this way, or by exposure to high levels of catecholamine after the death of the animal, recovery of the adrenergic response after isolation may occur. The earliest time at which catecholamine stimulation of the cells was carried out was 16 hours after initial isolation, at which point adrenaline was shown to stimulate cortisol production (Fig 3.4). Also, cells kept at $4^{O}C$ overnight (16-20 hours) failed to respond to catecholamines, but produced cortisol in response to $ACTH_{1-24}$, AII and carbachol. Beta-adrenergic receptors have variable half-lives, and reappearance after homologous desensitisation can take several days (Mahan *et al*, 1987). The argument that beta-adrenergic receptors are destroyed by the isolation

* Treatment of animals prior to death with adrenergic antagonists may theoretically stop this effect and may show the presence of an adrenergic response in freshly isolated cells. A single experiment showed that propranolol could cause desensitisation of the adrenergic response in day 3 cultured cells, so this approach may still be ineffective.

procedure also fits into this model as resynthesis of new receptors would follow the same time course as resensitisation if both involved protein synthesis.

Homologous desensitisation of catecholamine-stimulated steroidogenesis was also seen in cultured (day 3) cells (Fig 5.11). Desensitisation of responses mediated by beta-adrenergic receptors are common (Mahan, 1987) and are often associated with a short term conformational change in the receptor, reducing its ability to bind ligand, followed by a longer term degradation of receptor. Recovery of the cells from short term desensitisation would be expected to happen within minutes or hours, whereas resensitisation of the adrenergic response after receptor degradation would take a longer period. It is not known if the bovine ZFR cells studied followed this model.

The results presented in this chapter (most of which have been published as: Walker, Lightly, Milner & Williams, 1988) clearly show the presence of a steroid-producing beta-adrenergic response in cultured bovine ZFR cells. They also establish that this response is mediated by cyclic AMP and does not involve phosphoinositide-derived second messenger systems. Additionally, the response is subject to homologous desensitisation of unknown mechanism.

6 Further Study of the Beta Adrenergic Response

6.1 Introduction

The results in the previous chapter demonstrated that beta-adrenoceptors are responsible for mediating the effect of catecholamine stimulated steroidogenesis. Beta-adrenoceptors can be further classified into $beta_1$ and $beta_2$-receptors (Furchgott, 1972) and a recently characterised $beta_3$-subclass (Arch *et al, 1984;* Kaumann, 1989).

As discussed in section 1.6.2, there is some evidence for adrenergic innervation in certain species (Mikhail & Amin, 1969; Kleitman & Holzwarth, 1985), and this has been suggested as an alternative means of controlling adrenal steroidogenesis. In the bovine system there is no published data on this type of innervation. It is not absolutely clear yet whether there is actually adrenergic control *in vivo* in any species.

Ungar (1979) and O'Donnell (1987) have suggested that, in general, $beta_1$-adrenoceptors tend to be innervated by adrenergic neurons, whereas $beta_2$-adrenoceptors respond mainly to circulating catecholamines. $Beta_1$-adrenoceptors also have a lower affinity for catecholamines than $beta_2$-adrenoceptors, which is to be expected for receptors that are subject to much higher local concentrations of catecholamines in the synapse. Hence, determination of the subclass of beta-adrenoceptors on bovine ZFR cells should provide circumstantial evidence as to whether the adrenergic control of the adrenal cortex is by direct innervation or via circulating catecholamines.

Schild (Schild 1947a,b, 1949) first suggested the method of using pA_2 values, where:

$pA_2 = -\log_{10}$[dissociation constant of antagonist]

Initially, pA_2 values were used as a means for classifying antagonists and later this was extended to a method for differentiating between receptor subclasses (Kenakin, 1982). Previously, agonist potency ratios had been used (Lands *et al*, 1967a, b). However, this method is complicated by differing intrinsic efficacies of the agonists themselves. Schild analysis, as it is now called, eliminates this problem, relying only on the differing affinities of the antagonists used.

Subclassification of beta-adrenoceptors has been successfully reported using the selective $beta_1$-antagonist practolol (Dunlop & Shanks, 1968) and the selective $beta_2$-antagonist ICI118,551 (Bilski *et al*, 1983). These antagonists were used to determine the subclass of beta-adrenoceptors on the primary cultures of bovine adrenocortical ZFR cells used throughout this thesis. The effects of the selective beta-agonists; noradrenaline ($beta_1$), salbutamol ($beta_2$), dobutamine ($beta_1$), isoprenaline ($beta_{1/2}$), and BRL37344A ($beta_3$) were also compared.

6.2 Traditional Methods of Receptor Classification - An Introduction

Most of the detailed theory of drug-receptor pharmacology applicable to this chapter is contained in Kenakin (1987).

Several models have been derived to describe drug-receptor interactions in biological systems, one of the most important being the occupation theory which relies on equilibrium considerations for the derivation of the equations.

The action of an agonist on a cell or tissue depends on its ability to bind (affinity) and its capacity to elicit a response when bound (efficacy). The

total responsiveness is a combination of both of these factors. Study of agonist potency ratios was used originally to determine differences between agonist-receptor interactions - this being only a relative and not an absolute measure. However, though a single receptor subtype will produce the same potency ratios for a set of agonists in different tissues, the converse is not necessarily true ie. a set order of potency ratios does not imply a single receptor subtype. Further, differing efficacies of the agonists will produce false potency ratios: only full agonists - those that will produce the same maximum tissue response - can be used to classify receptors in this way. Partial agonists, producing only a fraction of the maximum response are less useful.

Ways exist to separate the affinity and efficacy contributions of the agonists. For example, using an irreversible antagonist in increasing concentration, progressively more of the receptors are immobilised, and extrapolation to 100% inactivation will give a measure of the agonist efficacy. These methods are, however, complex and a further step away from the *in vivo* situation.

A much more useful means of receptor classification is by determination of the affinity with which a competitive antagonist binds to a receptor. Antagonists have, of course, no efficacy contribution.

Schild (1947a,b, 1949) first proposed the use of pA_X values to classify antagonist-receptor interactions. pA_X is defined as *the negative (base 10) logarithm of the molar concentration of antagonist that produces an equiactive dose ratio of magnitude x* ie. pA_X represents a concentration of antagonist that produces an 'x' fold shift to the right of the agonist dose response.

A single antagonist will, in theory, have the same pA_X value when it

acts on a single receptor subtype in different tissues. Hence, determination of the pA_x value for a specific antagonist on the tissue in question, and comparison with previously published data for this antagonist, will give an indication as to whether that particular receptor is present on the tissue.

Derivation of the effects of a pure competitive antagonist on an agonist dose response using occupation theory yields the equation :-

$$\log_{10}(dr - 1) = \log_{10}[Antag] - \log_{10}(KAntag)$$

(Kenakin, 1987, p213)

where **dr** is the dose ratio, or the degree to which the antagonist shifts the agonist dose response to the right. **[Antag]** is the concentration of antagonist and K_{Antag} is the dissociation constant of the antagonist.

Schild regression analysis involves measuring the dose ratio for a series of antagonist concentrations and plotting $\log_{10}(dr - 1)$ versus $\log_{10}[Antag]$. This should give a straight line, **gradient = 1**, and **x-intercept** = $-\log_{10}(KAntag)$ for a pure competitive antagonist. A pA_2 value implies **dr = 2** and hence K_{Antag} = **[Antag]**, thus $pA_2 = pK_{Antag} = -\log_{10}(K_{Antag})$. Hence, it is possible to obtain a measure of the affinity of an antagonist for a receptor irrespective of the agonist used to get this result. Regressions with a gradient not equal to one, suggest that the antagonist is deviating from pure competitive behaviour. Reasons for this are discussed in Kenakin (1987).

Experimental determination of the pA_2 values of selective antagonists on a tissue or in a cell system gives a means of classifying receptor subtypes and also shows existence of non-equilibrium states. Study of agonist potency is a useful means of confirming or refuting the receptor classification using Schild analysis.

6.3 Statistical Analysis

For the estimation of antagonist pA_2 values, dose response curves were tested for parallelism by analysis of covariance using the SPSS-X statistical package produced by Edinburgh University Computing Service. Schild regression lines were fitted with a least squares fit using a Casio fx-180P programmable calculator and confidence limits calculated from the relevant statistical equations (Bowman & Rand, 1984) using tables of 2-tailed t statistics (Geigy Scientific Tables).

6.4 Results - The Effect of Various Selective Beta-Agonists on Cortisol Secretion

The effect of increasing concentrations of isoprenaline, noradrenaline, dobutamine, salbutamol and BRL37344A was tested on day 3 cells (Fig 6.1). The agonists had relative potency of:

isoprenaline > noradrenaline > dobutamine > salbutamol > BRL37344A.

Isoprenaline, noradrenaline and dobutamine all produced the same maximum response, salbutamol gave approximately 70% and BRL37344A gave approximately 10% of the maximum response.

Neither practolol nor ICI118,551 produced cortisol secretion by the cells, and therefore have no intrinsic agonist effects in these cells (Fig 6.2).

6.5 Schild Analysis - Determination of the pA_2 Values for 2 Selective Beta-Antagonists

Dose response curves (central portion shown is linear)* to isoprenaline were set up, in the presence of increasing doses of practolol or ICI118,551. Representative experiments for each antagonist are shown in Fig 6.3 & 6.4 (left-hand graphs). The dose response lines were judged to be parallel by

* curvature added by artist

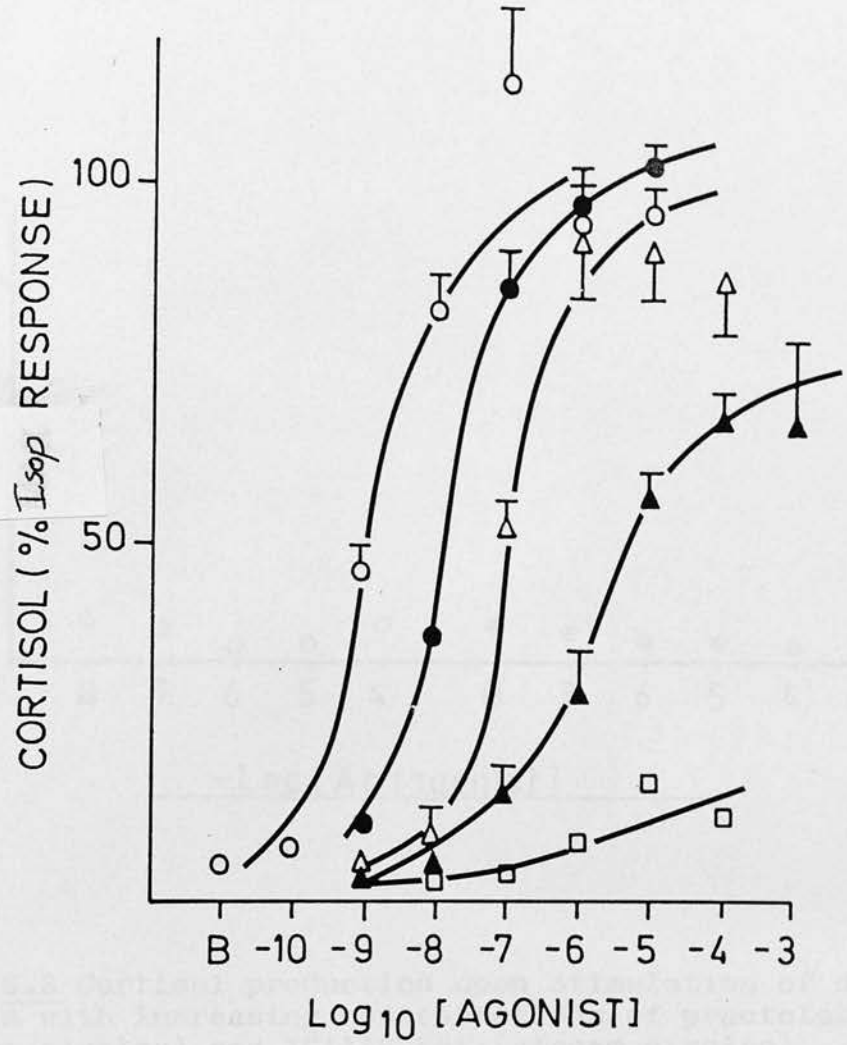


Fig 6.1 Dose-response curves for the secretion of cortisol produced upon stimulation of day 3 cells with isoprenaline (○), noradrenaline (●), dobutamine (△), salbutamol (▲) and BRL37344A (□). B = basal. Representative experiment (1 of 3 experiments).

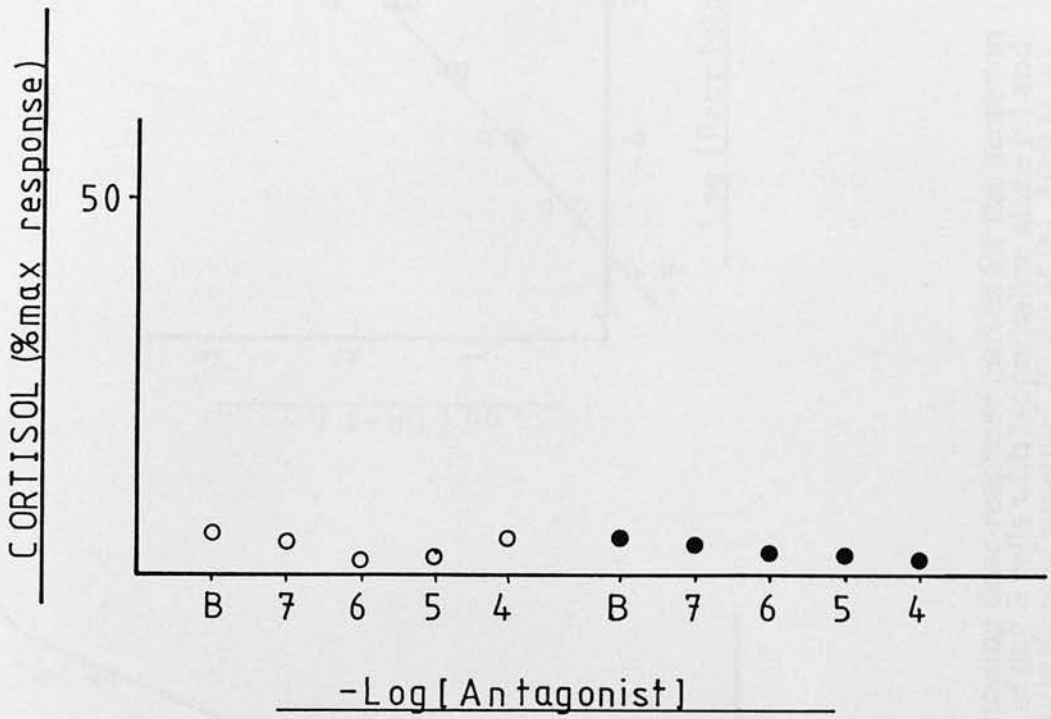


Fig 6.2 Cortisol production upon stimulation of day 3 cells with increasing concentrations of practolol (open circles) and ICI118,551 (closed circles). B = basal. Single experiment.

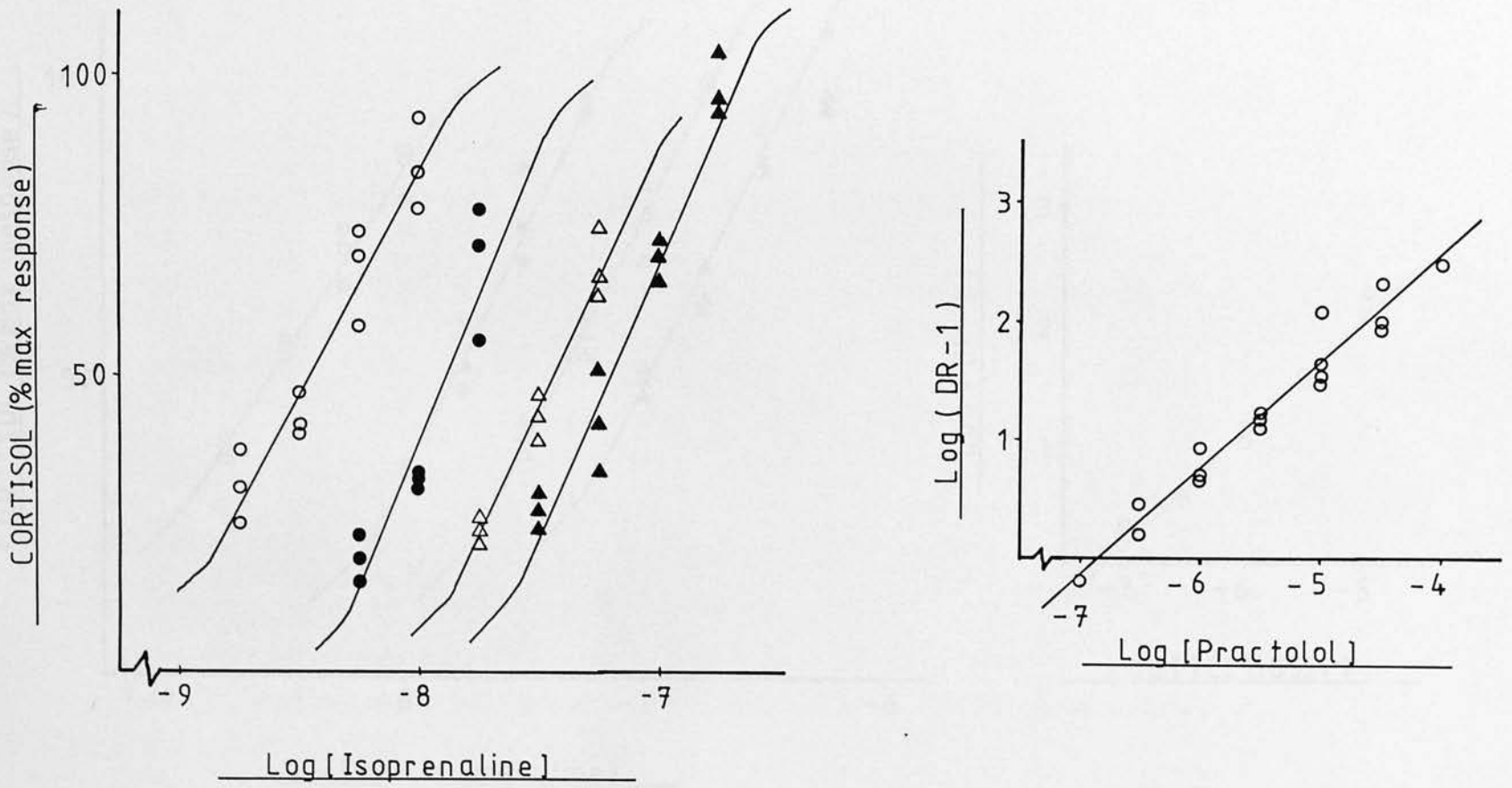


Fig 6.3 Representative experiment showing dose-response curves for the secretion of cortisol produced on stimulation of day 3 cells with isoprenaline alone (○) and in the presence of increasing concentrations of practolol $10^{-6.5}$ M (●), 10^{-6} M (△), $10^{-5.5}$ M (▲). Inset: Schild regression - least squares fit of Log_{10} (DR - 1) versus Log10 [practolol] where DR = dose ratio. Cumulative data from 4 separate experiments.

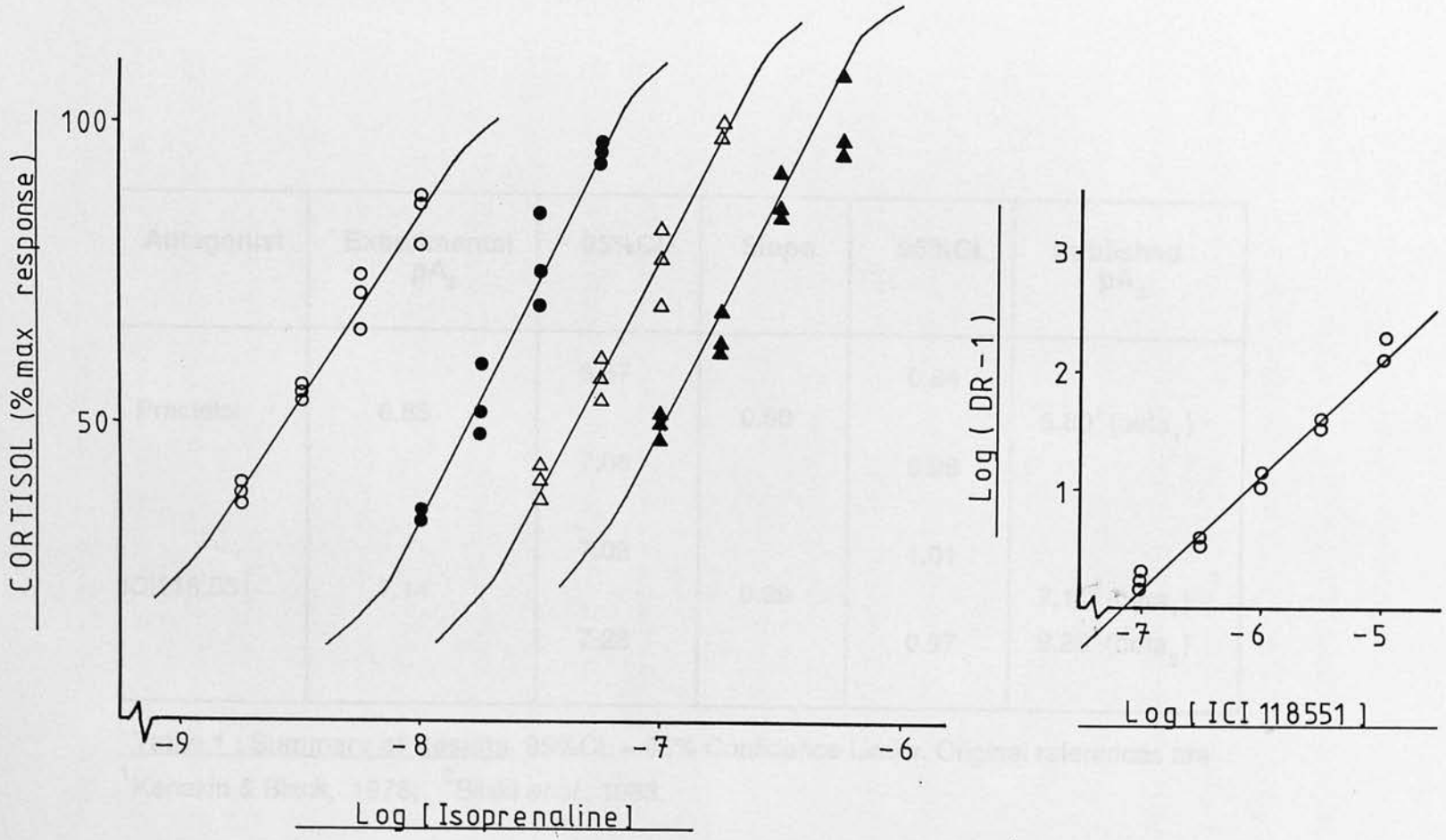


Fig 6.4 Representative experiment showing dose-response curves for the secretion of cortisol produced on stimulation of day 3 cells with isoprenaline alone (○) and in the presence of increasing concentrations of ICI118,551 $10^{-6.5}$ M (●), 10^{-6} M (△), $10^{-5.5}$ M (▲). Inset: Schild regression - least squares fit of Log_{10} (DR - 1) versus Log10 [ICI118,551] where DR = dose ratio. Cumulative data from 3 separate experiments.

Antagonist	Experimental pA_2	95%CL	Slope	95%CL	Published pA_2
Practolol	6.85	6.67 7.06	0.90	0.84 0.96	6.80[1] ($beta_1$)
ICI118,551	7.14	7.03 7.28	0.99	1.01 0.97	7.17[2] ($beta_1$) 9.26[2] ($beta_2$)

Table 1 : Summary of Results 95%CL = 95% Confidence Limits. Original references are [1]Kenakin & Black, 1978; [2]Bilski *et al.*, 1983.

analysis of covariance. Experiments were repeated on cells from 4 separate cell preparations for practolol and 3 separate cell preparations for ICI118,551.

The dose ratio for each concentration of antagonist was obtained and Schild plots of $\log_{10}(dr - 1)$ versus antagonist concentration plotted (Fig 6.3 & 6.4, right-hand graphs) and used to calculate pA_2 values for each antagonist and to obtain 95% confidence limits. Similarly, the slope of the regression line and 95% confidence limits were also estimated. Results for both antagonists are shown in Table I.

6.6 Discussion

The results presented in this chapter (and published as, Lightly, Walker, Bird & Williams, 1990) establish the existence of $beta_1$-adrenoceptors on cultured bovine ZFR cells.

Study of the effects of several selective beta-adrenergic agonists showed that isoprenaline, noradrenaline and dobutamine were all full agonists, whereas salbutamol acted as a partial agonist and was less potent than the full agonists. Salbutamol is known to act as a full agonist at $beta_2$-adrenoceptors, but only as a partial agonist at $beta_1$-adrenoceptors (Farmer *et al*, 1970). This in itself suggests that $beta_1$-adrenoceptors are present on the cells.

Although BRL37344 produced stimulation of the cells at 10^{-5} M, it was the least potent of all the agonists studied only producing 14% of the maximum response seen with isoprenaline. Previous studies have shown that, in systems known to contain $beta_3$-adrenoceptors, BRL37344 was a more potent agonist than isoprenaline (Arch *et al*, 1984; Bond & Clarke, 1988). Hence, it is likely that BRL37344 is producing a ~~non specific~~ weak

(possibly via $beta_1$-adrenoceptors)

stimulation and that $beta_3$-adrenoceptors are not present on cultured bovine ZFR cells.

Determination of the pA_2 values for the $beta_1$-antagonist practolol and the $beta_2$-antagonist ICI118,551 provided definitive evidence for the presence of $beta_1$-adrenoceptors (Table I). The pA_2 value for practolol of 6.85 (6.67 - 7.06) (Lower & upper 95% confidence limits) agreed satisfactorily with published values shown in the table. The gradient of the Schild regression was significantly less than 1, slope = 0.90 (0.84 - 0.96), suggesting deviation from an ideal competitive antagonist. Though practolol and ICI118,551 showed no partial agonist activity (Fig 6.2), practolol is known to be a partial agonist in other systems (Kenakin & Black, 1978). It is possible that isoprenaline may potentiate the partial agonist properties of practolol leading to production of more cortisol than expected and hence giving a Schild regression < 1.

The pA_2 value for the action of ICI118,551 at $beta_2$-adrenoceptors is 9.26 and at $beta_1$-adrenoceptors is 7.17 (Bilski *et al*, 1983). Hence, the experimental value of 7.14 (7.03 - 7.28) agrees with the value for $beta_1$-adrenoceptors. In this case the gradient of the regression line of 0.99 (0.97 - 1.01) suggests that ICI118,551 is acting as a pure competitive antagonist.

Thus, Schild analysis provided strong evidence that $beta_1$-adrenoceptors were present on the cells, and the results of the selective agonist stimulations confirmed this.

As mentioned in the introduction to this chapter $beta_1$-adrenoceptors are commonly found at adrenergic synapses. Thus it seems possible that there may be adrenergic innervation of the bovine adrenal cortex *in vivo*. Shima *et al* (1984) has already shown, using binding of [^{3}H]dihydroalprenolol, that membranes prepared from both the capsulated (ZFR) and

decapsulated (ZG) regions of the rat adrenal cortex contain $beta_1$-adrenoceptors, only the latter being coupled to adenylate cyclase *in vitro*.

Hence this evidence from the rat in which there is thought to be adrenergic innervation (Kleitman & Holzwarth, 1985), and the results presented in this chapter suggests that, at least in some species, adrenergic innervation may play a part in controlling steroidogenesis in the adrenal cortex.

7 The Cholinergic Response

7.1 Introduction

As discussed in section 3.3.4, during characterisation of the cell culture system, it was found that bovine ZFR cells secrete cortisol in response to stimulation with the cholinergic agonists, acetylcholine and carbachol. This was shown in both freshly isolated cells, and in cells between 2 - 5 days of primary culture.

Further analysis of the cholinergic response was carried out. An attempt was made to characterise the receptor subtype, and the second messenger system, responsible for mediating this response.

7.2 Initial Characterisation

As shown in Fig 3.6 (part of which is duplicated here as Fig 7.1 for convenience), the day-by-day responsiveness of the cells to ACh stimulation depended on the serum used in preparing the growth medium. In this representative experiment, the peak cortisol response (day 4) was seen in cells grown in CPSR5.

Earlier, (section 5.3) the measurement of cortisol, cyclic AMP and phosphoinositol levels in day 4 cells, stimulated with increasing concentrations of CCh, was described (Fig 5.6 is duplicated here as fig 7.2 for convenience). No significant increase in cyclic AMP was seen at any dose of CCh, while phosphoinositol production paralleled that of cortisol.

In section 4.4.1, the time-course of cortisol production in day 3 cells stimulated with 10^{-3} M CCh was studied (Fig 4.5). The time-course of phosphoinositol production in day 4 cells stimulated with 10^{-4} M ACh was

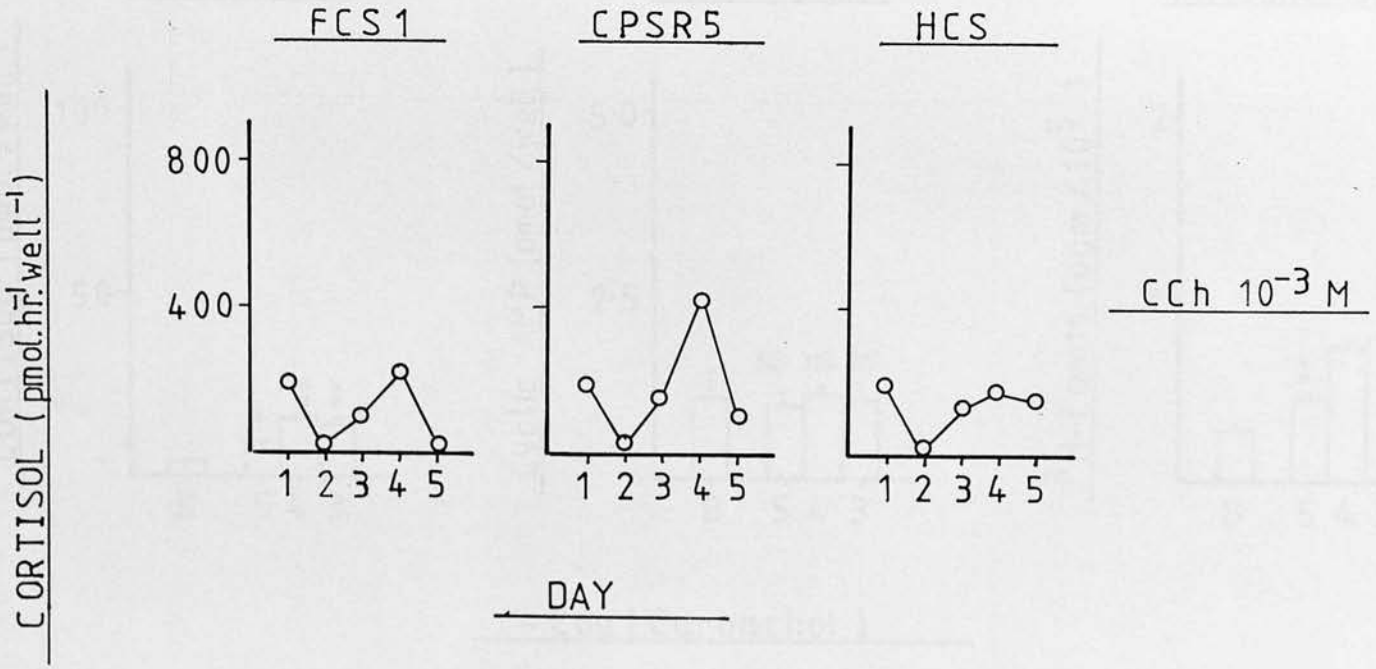


Fig 7.1 Cortisol produced by cells over a 1 hour period after stimulation with CCh (10^{-3} M) on each of the first 5 days of culture. Copy of part of Fig 3.6. Representative experiment (1 of 3 experiments).

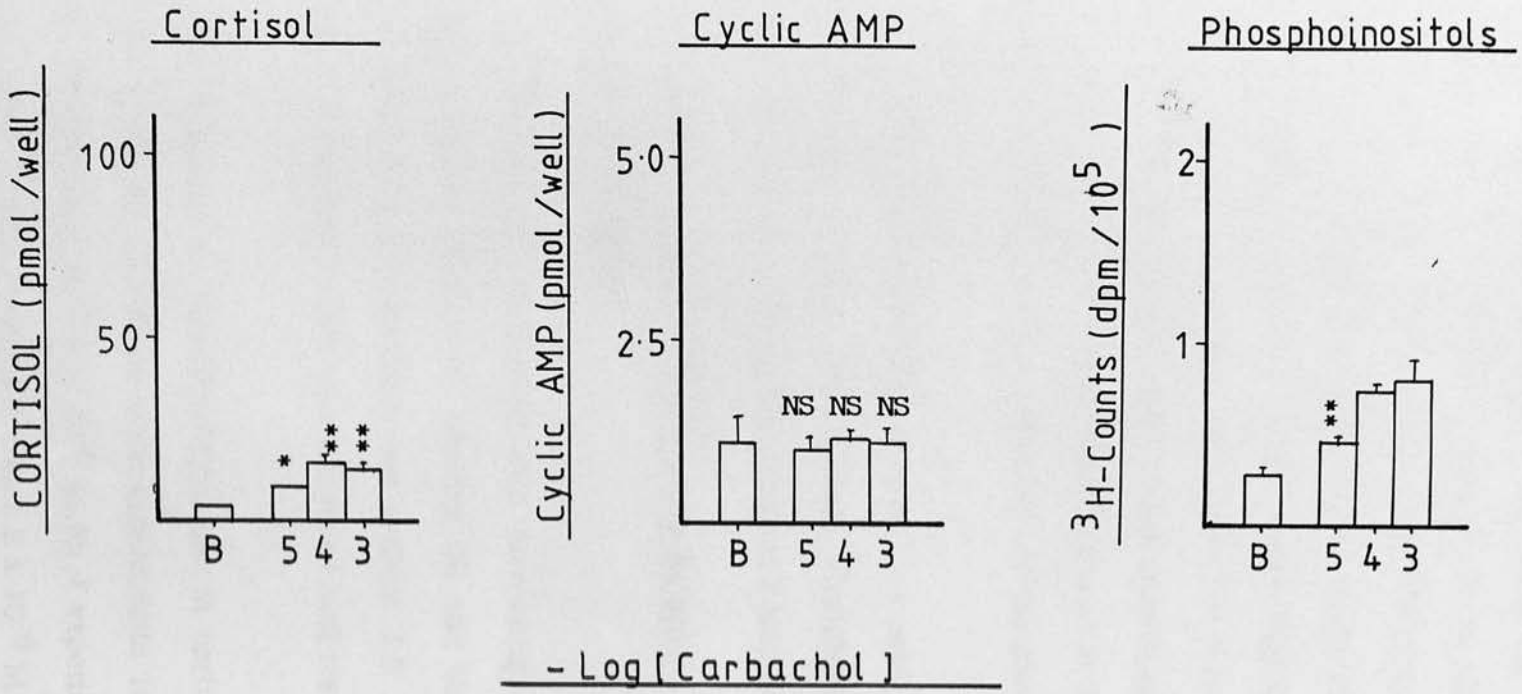


Fig 7.2 Cortisol, combined (medium + cellular) cyclic AMP and phosphoinositol production over a 15 minute time period in day 4 cells upon stimulation with increasing concentrations of CCh. Copy of Fig 5.6. Abbreviations are: NS, not significant; *, $p<0.05$; **, $p<0.01$; $n = 3$. B = basal. Representative experiment (1 of 3 experiments; results from the same experiment as Fig 5.4).

also measured (section 4.4.3, Fig 4.7).

Day 4 cells were stimulated with increasing concentrations of 10^{-4} M ACh and cortisol production measured. In parallel incubations, in the same batch of cells, but labelled with [^{3}H]-Inositol, phosphoinositol production was measured. Fig 7.3 shows the results obtained.

Basal cortisol output was almost linear. Significant levels of cortisol were not produced in stimulated cells until after 5 minutes ($p<0.001$ at 15 minutes). Stimulated cortisol output was non-linear, the output at 60 minutes being only slightly greater than that seen at 30 minutes. No further work on the possibility of desensitisation of the cholinergic response was carried out.

Phosphoinositol production of stimulated cells was linear for at least 15 minutes and subsequently declined. Phosphoinositol production in stimulated cells was statistically significant at 2 minutes ($p<0.005$).

7.3 The Effect of Selective Cholinergic Agonists and Antagonists on Cortisol Production

Day 3 cells were stimulated with increasing concentrations of the cholinergic agonists: ACh (M/N), nicotine (N) and McN-A-343 (M_1) (where M = muscarinic and N = nicotinic, see section 1.5 for details of known subtypes). Cortisol production was measured and results are shown in Fig 7.4.

ACh produced a dose-dependent rise in cortisol output, achieving maximum output by 10^{-4} M (in some experiments 10^{-5} M). The threshold response was between 10^{-7} and 10^{-6} M (in 4 experiments). The ED_{50} for ACh stimulated cortisol production was 1.2×10^{-6} M (Range: 3.7×10^{-7} to 3.7×10^{-6} M, in 4 experiments).

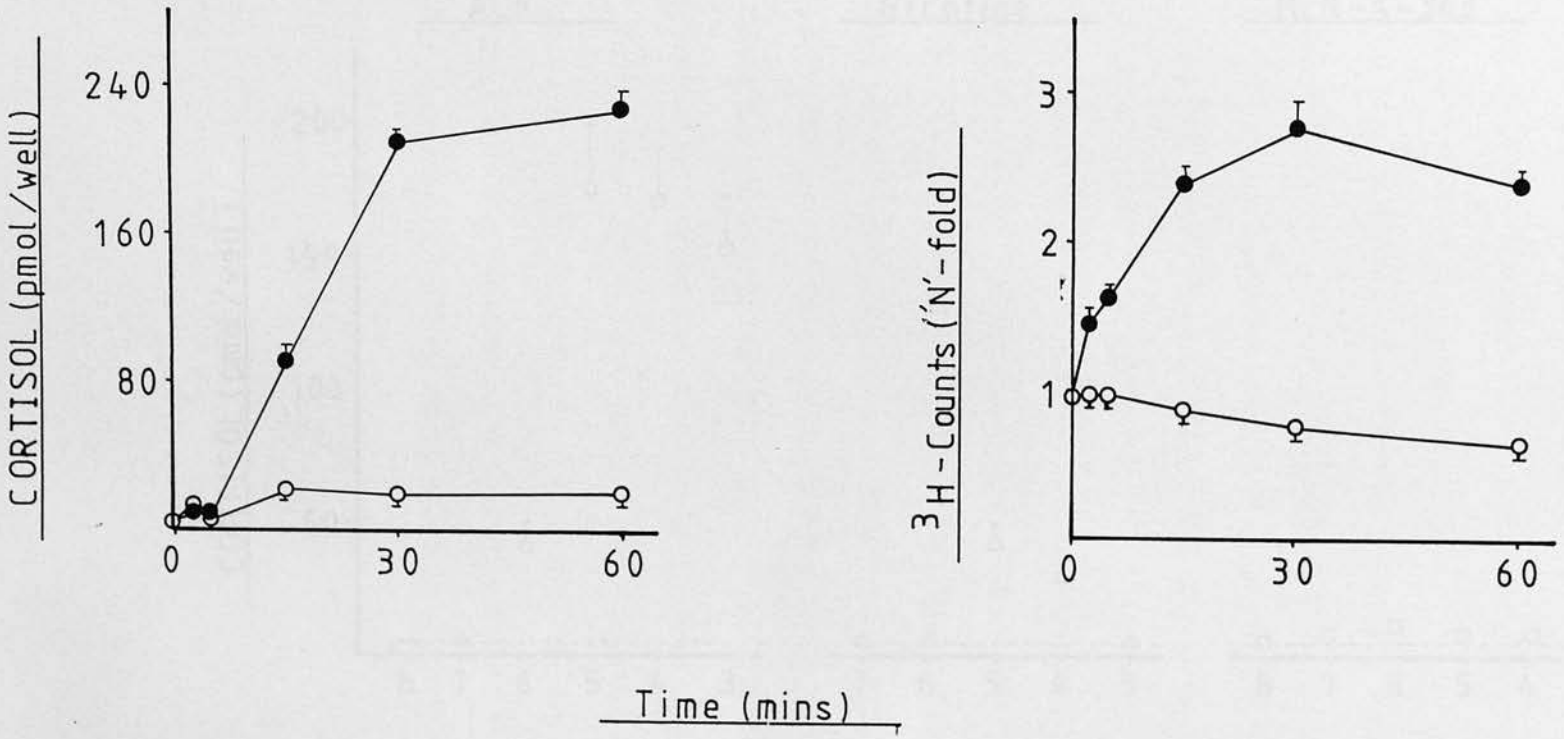


Fig 7.3 Time course of cortisol and phosphoinositol production measured in day 4 cells upon stimulation with ACh (10^{-4} M) (Closed circles). Open circles show basal values. Phosphoinositol production shown as 'N'-fold, where basal response = 1 at time = 0. Representative experiment (1 of 3 experiments).

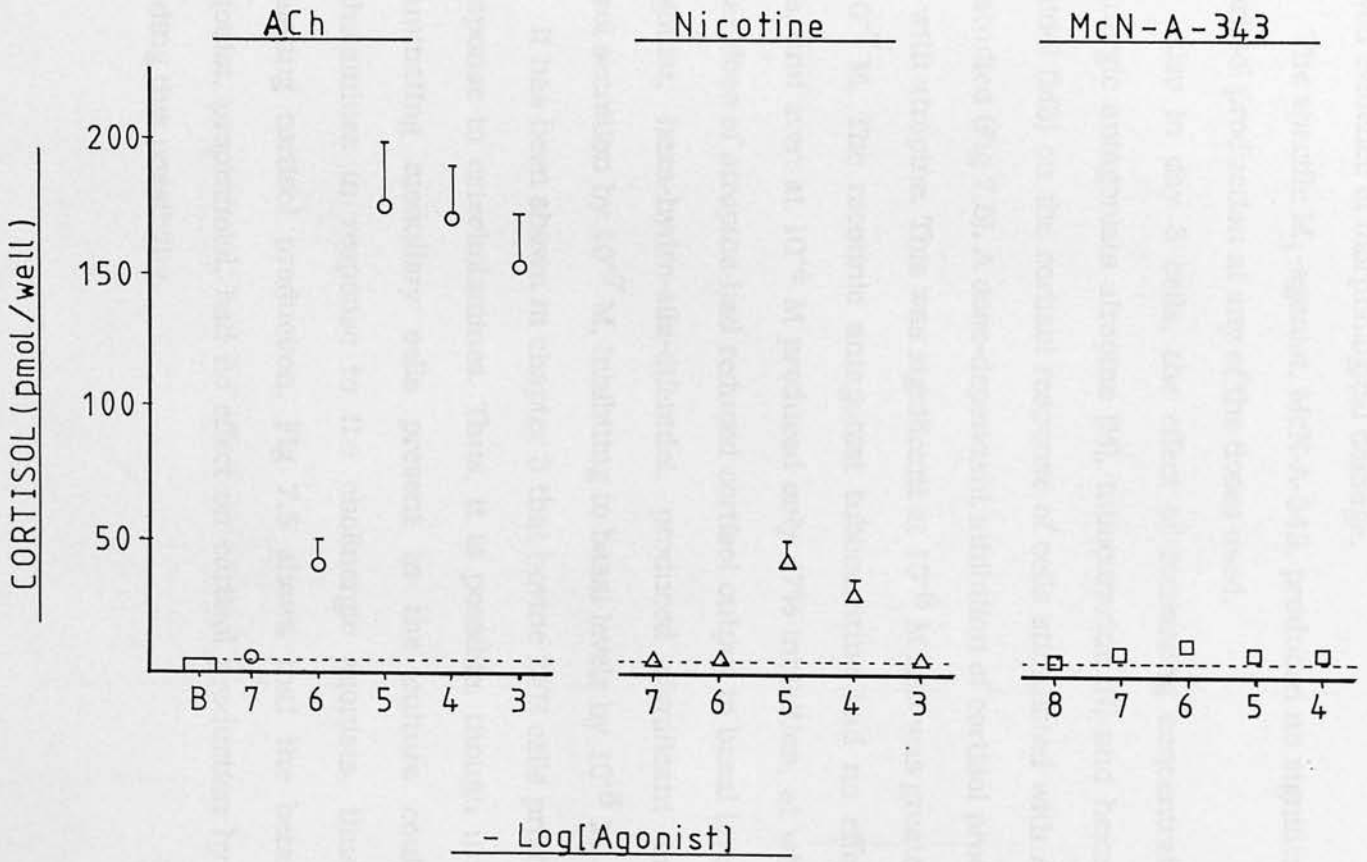


Fig 7.4 Cortisol produced by a 1 hour stimulation of day 3 cells with increasing concentrations of the cholinergic agonists shown. B = basal. Representative experiment (1 of 3 experiments, except nicotine results, which were repeated in 5 experiments).

Although nicotine stimulated cortisol production this was not seen until 10^{-5} M, and was then only 25% of the maximum seen with ACh. Stimulation with nicotine was also inconsistent, being seen in only 2 out of 5 experiments. Concentrations of nicotine > 10^{-5} M were less potent and cells showed evidence of morphological damage.

The specific M_1-agonist, McN-A-343, produced no significant increase in cortisol production at any of the doses used.

Also in day 3 cells, the effect of increasing concentrations of the cholinergic antagonists atropine (M), tubocurarine (N) and hexa-hydro-sila-difenidol (M3) on the cortisol response of cells stimulated with ACh 10^{-4} M was studied (Fig 7.5). A dose-dependant inhibition of cortisol production was seen with atropine. This was significant at 10^{-8} M and was greater than 60% by 10^{-7} M. The nicotinic antagonist tubocurarine had no effect at these doses, and even at 10^{-4} M produced only 47% inhibition, at which point a similar dose of atropine had reduced cortisol output to basal levels. The M_3-antagonist, hexa-hydro-sila-difenidol, produced significant inhibition of cortisol secretion by 10^{-7} M, inhibiting to basal levels by 10^{-5} M.

It has been shown in chapter 3 that bovine ZFR cells produce cortisol in response to catecholamines. Thus, it is possible, though unlikely, that contaminating medullary cells present in the culture could produce catecholamines in response to the cholinergic agonists, thus indirectly stimulating cortisol production. Fig 7.5 shows that the beta-adrenergic antagonist, propranolol, had no effect on cortisol production by ACh, thus excluding this possibility.

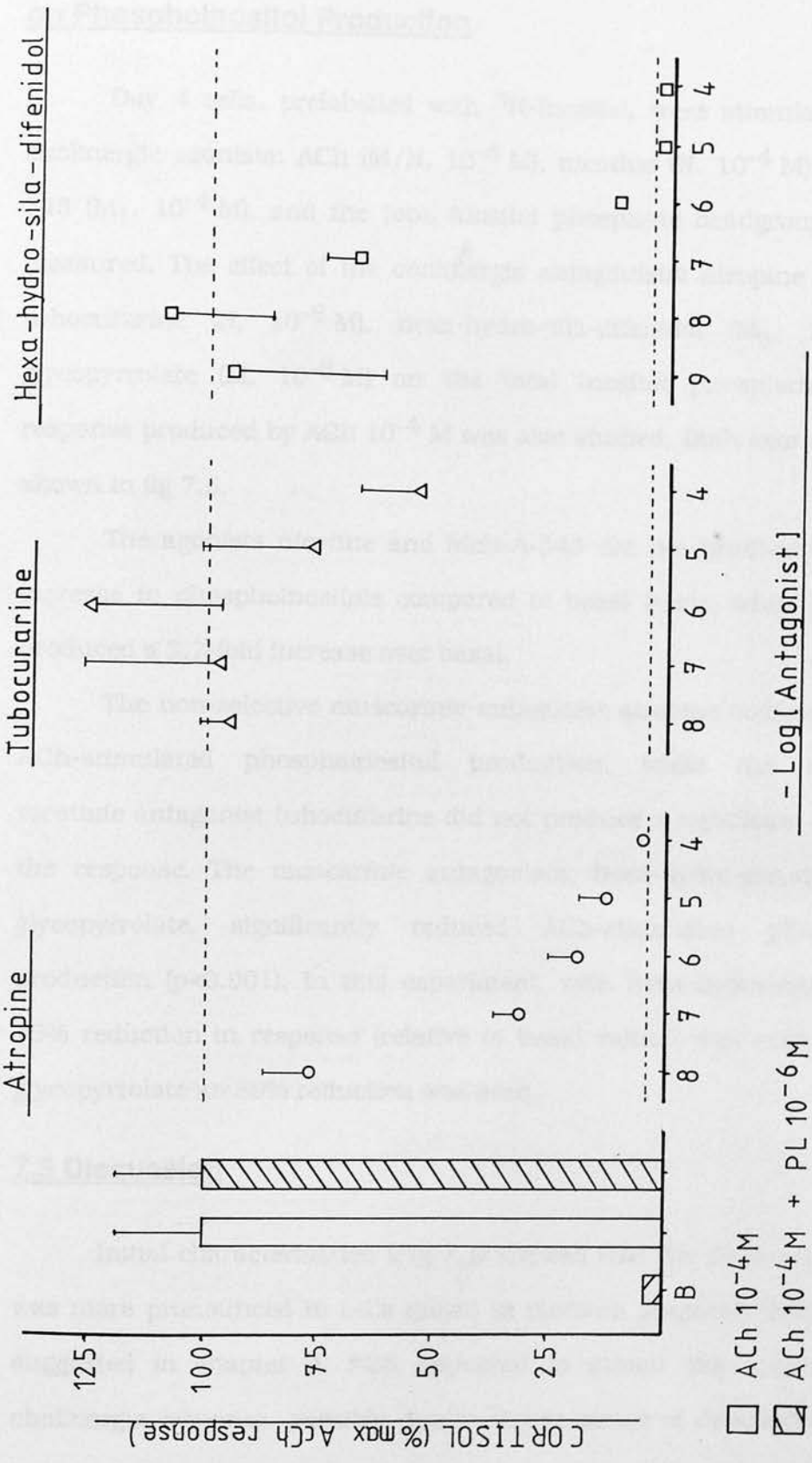


Fig 7.5 Cortisol produced by a 1 hour stimulation of day 4 cells. Left-hand bar graph shows basal and stimulated values produced by 10^{-4} M ACh, and the effect of propranolol (PL, 10^{-6} M) on ACh-stimulated cortisol production. B = basal. Graphs to right show inhibition of ACh-stimulated cortisol production produced by increasing concentrations of the cholinergic antagonists shown. Representative experiment (1 of 3 experiments).

7.4 The Effect of Selective Cholinergic Agonists and Antagonists on Phosphoinositol Production

Day 4 cells, prelabelled with ^{3}H-Inositol, were stimulated with the cholinergic agonists: ACh (M/N, 10^{-4} M), nicotine (N, 10^{-4} M) and McN-A-343 (M_1, 10^{-4} M), and the total inositol phosphate headgroup production measured. The effect of the cholinergic antagonists: atropine (M, 10^{-6} M), tubocurarine (N, 10^{-6} M), hexa-hydro-sila-difenidol (M_3, 10^{-6} M) and glycopyrrolate (M, 10^{-6} M) on the total inositol phosphate headgroup response produced by ACh 10^{-4} M was also studied. Both sets of results are shown in fig 7.6.

The agonists nicotine and McN-A-343 did not produce a significant increase in phosphoinositols, compared to basal levels, while ACh 10^{-4} M produced a 2.7-fold increase over basal.

The non-selective muscarinic antagonist atropine completely blocked ACh-stimulated phosphoinositol production, while the non-selective nicotinic antagonist tubocurarine did not produce a significant inhibition of the response. The muscarinic antagonists, hexa-hydro-sila-difenidol and glycopyrrolate, significantly reduced ACh-stimulated phosphoinositol production ($p<0.001$). In this experiment, with hexa-hydro-sila-difenidol a 75% reduction in response (relative to basal values) was seen, while with glycopyrrolate an 89% reduction was seen.

7.5 Discussion

Initial characterisation (Fig 7.1) showed that the cholinergic response was more pronounced in cells grown in medium prepared with CPSR5. As suggested in chapter 3, FCS appeared to inhibit the appearance of a cholinergic response, possibly due to the presence of complement proteins

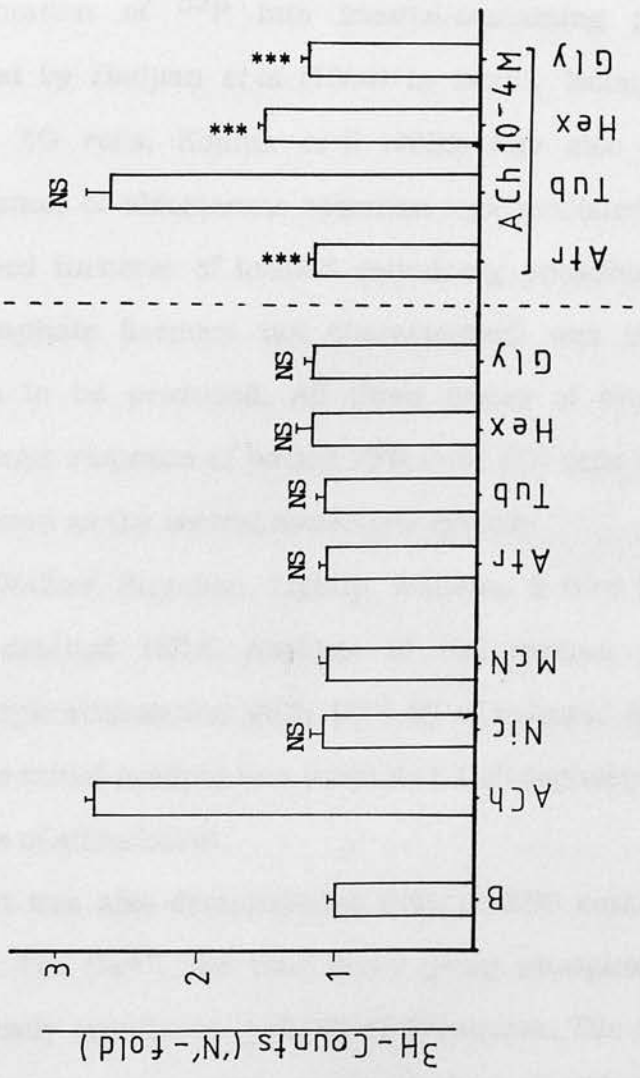


Fig 7.6 Total [^{3}H]-inositol phosphate headgroup response produced over a 15 minute period in day 4 cells upon addition of the cholinergic agonists and antagonists shown. Agonists (all present at 10^{-4}M): ACh - Acetylcholine; Nic - Nicotine; McN - McN-A-343. Antagonists (all present at 10^{-6} M): Atr - Atropine; Tub - Tubocurarine; Hex - Hexa-hydro-sila-difenidol; Gly - Glycopyrrolate. *Left-hand bars* show effects of agonists and antagonists relative to basal. *Right-hand bars* show inhibition of acetylcholine-stimulated phosphoinositol production by the antagonists shown. Results are shown as 'N'-fold relative to basal (=1). Abbreviations: NS, not significant; ***, $p<0.001$. Representative experiment (1 of 2 experiments).

normally removed by heat treatment of serum. In all experiments, freshly isolated (day 1) bovine ZFR cells produced cortisol in response to ACh or CCh. This had also been shown previously by Blair-West *et al* (1962) and Hadjian *et al* (1981, 1982). As with AII, cells on day 2 of culture showed a dramatically reduced response to cholinergic agonists (Fig 7.1).

The cholinergic response of the cells is mediated by a process involving increased turnover of inositol-containing phospholipids and not involving changes in cyclic AMP concentration (Fig 7.2). Increased incorporation of ^{32}P into inositol-containing phospholipids was also reported by Hadjian *et al* (1984) in freshly isolated bovine ZFR cells. In bovine ZG cells, Kojima *et al* (1986) has also shown that cholinergic stimulation of aldosterone secretion was mediated by a process involving increased turnover of inositol containing phospholipids, and that inositol trisphosphate (isomers not characterised) was the first phosphoinositol species to be produced. All these pieces of evidence suggest that the cholinergic response of bovine ZFR (and ZG) cells utilises phosphoinositide breakdown as the second messenger system.

Walker, Strachan, Lightly, Williams & Bird (1990) have also shown, using detailed HPLC analysis of the inositol phosphate products of cholinergic stimulation (ACh 10^{-4} M) of cultured (day 4) bovine ZFR cells, that the initial product was inositol (1,4,5)trisphosphate, appearing within 5 seconds of stimulation.

It was also demonstrated that, in EBS containing the equivalent of 120nM free Ca^{2+}, the total head group phosphoinositol production was statistically significant ($p<0.05$) at 5 minutes. The response was 19.25% of that seen in normal EBS. Basal labelling at 120nM Ca^{2+} was unaffected. This reduction in response is similar to that seen with AII in the same cells

(Bird *et al*, 1989).

Both the appearance of inositol(1,4,5)tris phosphate as the initial product of ACh stimulation, and the presence of a response in low Ca^{2+} buffer are consistent with the activation of phospholipase C by cholinergic agonists.

Walker, Strachan, Lightly, Williams & Bird (1990) found that, in bovine ZFR cells kept in suspension until day 4 and pre-loaded with the calcium indicator Fura-2, ACh (10^{-4} M) produced a 1.6-fold increase in intracellular [Ca^{2+}]. Under similar conditions, AII produced a 1.8-fold increase in intracellular [Ca^{2+}]. Atropine completely blocked the rise in intracellular [Ca^{2+}] produced by Ach, but had no effect on the increase caused by AII. Again, in buffer containing 120nM Ca^{2+}, the increase in intracellular [Ca^{2+}], though reduced, was still present for both agonists. These observations confirm that release of intracellular Ca^{2+} is involved in mediating the response of bovine ZFR cells to cholinergic agonists. This is consistent with phospholipase C activation as the primary response.

The results presented in this chapter provide evidence that M_3-cholinergic receptors are responsible for mediating steroidogenesis stimulated by cholinergic agonists in primary cultures of bovine ZFR cells. Thus, Fig 7.4 shows that ACh was a potent stimulant of steroidogenesis in the bovine ZFR cells, and that the M_1-selective agonist, McN-A-343, had no effect. While nicotine stimulated steroidogenesis in 2 out of 5 experiments, this was only to 20% of the levels of ACh. This suggests that nicotine may be acting non-specifically to stimulate steroidogenesis. Nicotine was also shown to be cytotoxic at doses above 10^{-5} M. These results confirm those of Hadjian *et al* (1981, 1982), that muscarinic receptors are present on bovine adrenocortical ZFR cells. Benjamina *et al* (1987) has shown that muscarinic

receptors are responsible for mediating the steroidogenic response to cholinergic agonists in the frog, although Rubin & Warner (1975) have shown that nicotine stimulates steroidogenesis in a mixed adrenocortical preparation from the cat.

It was also shown that the muscarinic antagonist, atropine, caused a dose-dependent blockade of steroidogenesis stimulated by 10^{-4} ACh. The nicotinic antagonist, tubocurarine, produced antagonism at doses above 10^{-6} M. The M_3-antagonist, hexa-hydro-sila-difenidol, also showed a dose-dependent blockade of steroidogenesis. It is not possible to say conclusively from these results whether tubocurarine is simply acting non-specifically on muscarinic receptors, but as it is much less potent (in the order of 100 times) than either of the muscarinic antagonists, it would seem likely that only muscarinic receptors are present. To determine the subtype of receptor conclusively requires the use of Schild analysis as described for the beta-adrenergic receptor in chapter 6 and possibly additional evidence from radio-ligand binding studies.

The effect of these agonists and antagonists on the phosphoinositol response was also studied (Fig 7.6), showing that nicotine and McN-A-343 had no effect, while ACh produced a 2.7-fold increase relative to basal. All of the antagonists studied had no intrinsic effect on phosphoinositol turnover. Again it was shown that tubocurarine had no significant effect on phosphoinositol turnover, while the M_3-selective antagonist, hexa-hydro-sila-difenidol produced considerable inhibition of the phosphoinositol response. Glycopyrolate, which is more selective antagonist for M_3 receptors, but is also effective as an M_1-antagonist, also caused considerable inhibition of the phosphoinositol response produced by Ach.

~~The evidence that cortisol production, stimulated by cholinergic~~

agonists, from primary cultures of bovine adrenocortical ZFR cells is mediated by a phospholipase C dependent mechanism, is consistent with the presence of M_3 cholinergic receptors on the cells, as M_1 and M_3, but not M_2 cholinergic receptors have been shown to be linked to phosphoinositide turnover (Fukuda *et al*, 1988).

8 Conclusions

In addition to a detailed description of the characterisation of a primary culture system for bovine adrenocortical zona fasciculata / reticularis cells, this thesis aimed to address the two questions stated in the introduction:-

(1) What relevance do adrenergic and cholinergic stimulation of cortisol production have to the overall, currently accepted, picture of regulation of cortisol production in the adrenal zona fasciculata / reticularis cells?

(2) What molecular mechanisms are involved in adrenergic and cholinergic stimulated cortisol production in bovine zona fasciculata / reticularis cells, and how do these relate to the mode of action of other adrenocortical secretagogues?

8.1 Characterisation of a Cell Culture System

The need to use a purified adrenocortical cell preparation has been emphasised in the reviews of Tait *et al* (1980) and Brown *et al* (1982). Even minor contamination by cells from other zones may influence experimental results. Many other workers who have used enzymically dispersed adrenocortical cells have either worked with a mixed adrenocortical cell preparation (Kawamura *et al*, 1984, 1985) or have used cells prepared from dissected tissue (Gospodarowicz *et al*, 1977). The preparation employed to isolate the cells used in the experiments contained in this thesis relied on an initial primary separation of the zones by slicing of the gland using a Staddie-Riggs microtome and selection of tissue slices containing only zona fasciculata cells. Additionally the purification method

of McDougall, Williams *et al* (1979) was employed. This yielded cells essentially free from red blood cells and debris. Cells were also free from glomerulosa cell contamination, as AII-stimulation failed to show aldosterone production (Williams, Lightly, Ross, Bird & Walker, 1988). The cells responded steroidogenically to $ACTH_{1-24}$ and AII both before and after several days in culture. Thus, a viable cell isolation and culture system for bovine zona fasciculata / reticularis cells was used to obtain the results contained in this thesis.

8.2 Question (1) - Relevance of Adrenergic and Cholinergic Mechanisms

There is already evidence for innervation of the adrenal cortex in several species (Section 1.4.1), but no clear knowledge of any effect of adrenergic agonists *in vivo*. The results in chapter 3 show that freshly isolated, enzymically dispersed, cells do not respond steroidogenically to adrenergic agonists. This finding is in agreement with the findings of Sequira & McKenna (1985), Kawamura *et al* (1984) and DeLean *et al* (1985). In addition, cells respond steroidogenically to adrenergic agonists after approximately 12-18 hours of primary culture. This was also seen by Kawamura *et al* (1984) in a mixed bovine adrenocortical preparation, and by DeLean *et al* (1985) in cultured rat subcapsullar cells.

Shima *et al* (1984) showed, using binding of[^{3}H]-dihydro-alprenolol, that rat adrenal cortex possessed $beta_1$-adrenoceptors. In chapter 5, it was shown that catecholamine stimulation of steroidogenesis in cultured bovine ZFR cells was mediated by beta-adrenoceptors, and further in chapter 6 that these receptors were of the $beta_1$-subtype. As discussed in chapter 6, $beta_1$-adrenoceptors tend to be associated with innervated tissue with the implication that adrenergic innervation of the adrenal cortex may be linked

to steroid secretion *in vivo*.

Physiologically, one potential advantage to mammals of using adrenergic innervation to stimulate steroidogenesis, would be that the response would be almost instantaneous. Although stimulation of adrenocortical steroidogenesis by ACTH[*1] is central to the mammalian stress response, it is possible that the initial stimulus is via adrenergic innervation.[*2]

In chapter 7 the cholinergic response of bovine adrenal ZFR cells was discussed. It was shown that freshly isolated, enzymically dispersed, cells responded steroidogenically to cholinergic agonists, supporting the evidence of Blair-West *et al* (1962) in freshly isolated sheep adrenocortical cells, Rubin & Warner (1975) in cat cells, and Hadjian *et al* (1981, 1982, 1984) in bovine adrenocortical cells. The steroidogenic response was also seen in cultured cells, in agreement with the results of Kawamura *et al* (1985) who showed that primary cultures of a mixed preparation of bovine adrenocortical cells responded steroidogenically to acetylcholine. Additionally it was shown (chapter 7) that cholinergic-stimulated steroidogenesis in bovine zona fasciculata / reticularis cells was mediated by M_3-cholinoceptors.

An important observation is the occurrence of cholinergic stimulation of steroid production in freshly isolated cells, which suggests that the cells have the potential to respond *in vivo*. This finding makes it difficult to argue that the cholinergic response is an artefact of culture. As there is evidence for cholinergic innervation of the adrenal cortex (Coupland, 1961; Robinson *et al*, 1977), this is yet further evidence for the presence of a cholinergic response of adrenocortical cells *in vivo*.

*$_1$ From stimulation of CRH release from the hypothalmus, to the direct action of ACTH on the adrenal cortex would be expected to take several minutes, whereas innervative control could theoretically act in less than a second.

*$_2$ by direct innervation of the adrenocortical cells, in addition to innervation of blood vessels, and control of adrenal blood supply.

8.3 Question (2) - Mechanisms of Adrenergic and Cholinergic Agonists

The results presented in chapter 5, showed clearly that adrenergic stimulation of adrenal steroidogenesis in the cells was mediated solely by cyclic AMP production. This is consistent with the presence of $beta_1$-adrenoceptors on the cells. This provides a useful method for further investigating the effects of cyclic AMP on steroidogenesis.

It is not known whether ACTH receptors are linked to adenylate cyclase via a G protein. If this is the case, it is possible that the $beta_1$-adrenoceptors present on the cells link to the same adenylate cyclase units via a different G protein or link to both the same adenylate cyclase and G protein units. Alternatively the adenylate cyclase / G protein units may be entirely separate. A simple test of additivity of ACTH and adrenaline may have helped to clarify this point.

Further studies, for example, identifying the various G proteins in the cells by specific G protein antibodies which it is now possible to produce, would also be a useful means of identifying any link between the ACTH and adrenergic effector systems. It should be possible to study the properties of the adenylate cyclase units which are present in the cells by isolating bovine zona fasciculata // reticularis cell membranes from cultured cells, stimulating with ACTH or adrenergic agonists and assaying for adenylate cyclase by one of the standard assay procedures. Using this assay, the kinetic and other properties of the adrenergic and ACTH-linked adenylate cyclase systems could be compared

It seems likely that adrenergic agonists stimulate steroidogenesis in a similar manner to ACTH // cyclic AMP -stimulated steroidogenesis. In contrast, cholinergic agonists appear to stimulate a phospholipase C, increasing levels of inositol 1,4,5-tris phosphate (and other inositol

phosphates), and thereby to cause raised intracellular [Ca^{2+}]. Cholinergic agonists do not appear to produce any increase in cellular cyclic AMP levels. This process shares much in common with AII-stimulated steroidogenesis, though the magnitude of the PI response, and the amount of steroid produced, is much less (Bird *et al.*, 1989).

Thus, cholinergic agonists and AII appear to stimulate steroidogenesis in a similar manner. They both cause activation of phospholipase C and an increase in intracellular [Ca^{2+}]. This is likely to lead to the stimulation of a calmodulin-dependant protein kinase and possible activation, by phosphorylation, of several steroidogenic enzymes. Again further work is needed to clarify this, for example by analysis of the particular protein kinases present in the cells by the use of phosphorylation assays. The proteins that these kinases act on could also be identified.

Thus the second messenger systems activated by ACTH and adrenergic agonists, and AII and cholinergic agonists are similar.

8.4 Summary of Aims

The questions posed at the beginning of this thesis, though not fully answered, have been given full consideration. A primary culture system for bovine adrenocortical zona fasciculata / reticularis cells has been established, the relevance of the adrenergic and cholinergic response of adrenocortical cells has been discussed, and the mechanisms of action of these effectors has been elucidated.

Appendix I

Serum used in Experiments Presented in Figures

FCS

3.1; 4.1; 4.2; 4.3; 5.1; 5.2; 5.3; 5.7; 5.8; 5.10; 5.11.

CPSR5

4.4; 4.5; 4.6; 4.7; 5.4; 5.6; 5.9; 6.1; 6.2; 6.3; 6.4; 7.2; 7.3; 7.4; 7.5; 7.6.

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Characterization of the steroidogenic responsiveness and ultrastructure of purified zona fasciculata/reticularis cells from bovine adrenal cortex before and after primary culture

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ABSTRACT

Analysis by electron microscopy indicated that after 3 days of primary culture, purified bovine adrenal zonal fasciculata/reticularis (ZF/ZR) cells showed improved integrity of their ultrastructure, with an increased density of lipid droplets and smooth endoplasmic reticulum. The basal cortisol output was significantly ($P<0{\cdot}05$) greater on day 3 of culture than for the freshly isolated cells in six out of seven experiments. Similarly, in six experiments with ACTH (1 nmol/l) and five experiments with angiotensin II (10 nmol/l), the stimulated cortisol secretion was significantly ($P<0{\cdot}01$ for all 11 experiments) higher on day 3 of culture than in freshly isolated cells.

No significant increase in cortisol secretion above basal was observed with noradrenaline at any concentration in the freshly isolated cells, whereas a dose-dependent increase in cortisol secretion was observed on day 3 of culture in all of four experiments. These findings were supported by cyclic (c) AMP output measured in one such experiment. Thus the basal cAMP output and that stimulated by ACTH (1 nmol/l) were significantly higher after culture ($P<0{\cdot}001$, $n=$ five wells for basal comparison; $P<0{\cdot}05$, $n=$ three wells for ACTH at 1 nmol/l). In agreement with the cortisol results, cAMP production was unaffected by any concentration of noradrenaline in the freshly isolated cells, whereas a dose-dependent rise was found after culture. Angiotensin II at all concentrations had no effect on cAMP production in freshly isolated or cultured cells.

These studies demonstrate that the primary culture of purified bovine ZF/ZR cells increases basal steroidogenesis and leads to an enhanced responsiveness to the physiological stimuli, ACTH and angiotensin II. In addition, primary culture of purified bovine ZF/ZR cells revealed a noradrenaline response which was not readily observed in freshly isolated, purified ZF/ZR cells.

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INTRODUCTION

Since the enzymic digestion procedure was first employed to obtain dispersed adrenocortical cells (Kloppenborg, Island, Liddle *et al.* 1968; Swallow & Sayers, 1969), the use of isolated zona glomerulosa (ZG), zona fasciculata (ZF) and zona reticularis (ZR) cells has considerably advanced our understanding of the control mechanisms which regulate the secretion of adrenal steroids. The need to use purified cell preparations for these studies has been stressed in two major reviews (Tait, Tait & Bell, 1980; Brown, 1982), and several methods are now available for the purification of rat ZG, ZF and ZR cells (Tait, Tait, Gould & Mee, 1974; Bell, Gould, Hyatt *et al.* 1978; McDougall, Williams, Hyatt *et al.* 1979; Tait *et al.* 1980; Chu & Hyatt, 1986).

Bovine adrenocortical cells from the inner zones have not been rigorously purified, unlike cells from the rat. Workers have employed either unpurified freshly isolated cells prepared from adrenocortical slices (Hepp, Grillet, Peytremann & Vallotton, 1977) or unpurified cultured cells prepared from crudely dissected inner-zone adrenal cortical tissue (Gospodarowicz, Ill, Hornsby & Gill, 1977). In order to obtain a reproducible isolated ZF/ZR cell system to study the intracellular mechanisms which are involved in cortisol secretion, we have recently employed the column

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filtration method (McDougall *et al.* 1979) for the purification of ZF/ZR cells from the bovine adrenal cortex. The steroidogenic capacity of these purified ZF/ZR cells has been compared with the same cells after 3 days in primary culture using adrenocorticotrophic hormone (ACTH), angiotensin II (AII) and noradrenaline as stimulants.

MATERIALS AND METHODS

Bovine adrenal glands were obtained from steers (age range 18 months to 2 years) within 20 min of slaughter at the Gorgie Abattoir, Edinburgh and transported within 20 min to the laboratory on ice. After trimming away the fatty tissue under sterile conditions, the glands were rinsed with Earle's balanced salt solution (EBSS; Gibco Biocult Ltd, Paisley, Strathclyde, U.K.) and placed in a Staddie-Riggs microtome.

Slices of tissue of approximately 100 μm thickness were cut using a skin graft blade. The first slice, which consisted of the adrenal capsule and adhering zona glomerulosa, was discarded and the second and third slices which contained the ZF/ZR (with no visible contamination by the adrenal medulla) were finely chopped into 1–2 mm portions and digested by incubation with collagenase (Worthington Biochemical Corporation, Lorne Diagnostics Ltd, Bury St Edmonds, Suffolk, U.K.) dissolved in EBSS (1·5–2·0 mg/ml) containing 2% (w/v) bovine serum albumin (BSA, fraction V; ICN Biomedical Ltd, High Wycombe, Bucks, U.K.). Routinely, 3 g adrenal ZF/ZR tissue were digested for 90 min in 30 ml collagenase solution in a shaking water bath at 37 °C; at 30-min intervals the tissue was dispersed mechanically (Haning, Tait & Tait, 1970) by repeated pipetting (30 strokes, 5 ml pipette).

Dispersed cells were separated from undigested tissue by filtration through a 250 μm mesh nylon gauze (Henry Simon, Stockport, Cheshire, U.K.). The cells were then harvested by centrifugation at 300 *g* for 15 min at 4 °C, washed three times in EBSS containing 0·2% BSA and filtered through 100 μm mesh nylon gauze followed by 30 μm mesh nylon gauze; this removed any aggregates present. The cell suspension (10 ml) was then applied to a Sephadex column (McDougal *et al.* 1979) which consisted of 5 ml G-50 Sephadex (Pharmacia, Milton Keynes, Bucks, U.K.) supported on 5 ml G-10 Sephadex; this had previously been equilibrated with EBSS containing 0·2% BSA at room temperature.

A further 20 ml EBSS containing 0·2% BSA was passed through the column to remove red blood cells, cell debris and small damaged cells. The ZF/ZR cells were then harvested from the column by resuspending the Sephadex and filtering through a 30 μm nylon gauze; this allowed the adrenal cells to pass through, but not the Sephadex beads (McDougall *et al.* 1979). From the original unpurified cell population, approximately 70% of the ZF/ZR cells were retained by the Sephadex as judged by light microscopy, and more than 90% of these cells were considered viable by the trypan blue exclusion test. Routinely 3 g ZF/ZR tissue gave a yield of approximately 15×10^6 purified ZF/ZR cells, essentially free from cell debris and red blood cells.

After counting the cells in an improved Neubauer haemocytometer, they were either used directly to study steroid secretion or were plated out in 12-well plates in Ham's F12 medium containing 10% (v/v) fetal calf serum (NBL, Cramlington, Northumberland, U.K.) with added penicillin (50 IU/ml), streptomycin (50 μg/ml) and amphotericin B (2·5 μg/ml) (Flow Laboratories, Rickmansworth, Herts, U.K.) using 2×10^5 cells per well. The cells were cultured at 37 °C in an atmosphere of 5% CO_2, and the medium was changed on the second day. Steroid secretion from the cultured cells was studied on the third day, except for the first series of experiments which evaluated the steroid output of the cells daily from days 1 to 5 of culture.

Incubation of dispersed cells

Freshly isolated purified ZF/ZR cells were incubated for 1 h at 37 °C in 1 ml EBSS containing 0·2% BSA in the presence of increasing concentrations of ACTH(1–24) (Synacthen; CIBA Laboratories, Horsham, Sussex, U.K.; 10 fmol–10 nmol/l), [Asp^1,Val^5]-angiotensin II (MRC Research Standard A; NIBSC, Hampstead, London; 0·01–10 nmol/l) or noradrenaline (Winthrop Laboratories, Horsham, Sussex, U.K.; 1 nmol–1 μmol/l). All incubations were carried out in triplicate for each agonist concentration and a basal secretion (no agonist) measured for each experiment; at least three experiments were performed with each agonist. At the end of the incubation period the cell suspensions were centrifuged at 300 *g* for 15 min at 4 °C and the cyclic AMP (cAMP), cortisol and aldosterone (in some experiments) outputs measured in the medium by radioimmunoassay procedures. In addition, cAMP was also measured in an ethanol extract of the cell pellets.

Cultured purified ZF/ZR cells were initially washed once with EBSS containing 0·2% BSA, after removing the tissue culture medium. However, the relatively long period of basal cortisol secretion during culture led to significant carry-over of cortisol into the incubation medium when only one wash was used. Consequently, with the exception of the earlier experiments analysing the day-by-day response, all later experiments used two washes with EBSS plus 0·2% (w/v) BSA. Incubations were then carried out exactly as

This difference is, therefore, unlikely to be due solely to limited availability of substrate for steroidogenesis in freshly isolated cells. Instead, the greater responsiveness may be due to an increase in the number of viable receptors for ACTH in the cultured cells, with activation of more adenylate cyclase units within the plasma membrane. Alternatively, there may be an increase in the number of adenylate cyclase units capable of coupling to the receptors, since only a small proportion of ACTH receptors need to be activated in order to obtain maximal steroidogenesis (McIlhinney & Schulster, 1975). It should be possible to determine which of these explanations is correct by performing binding studies using biologically active ^{125}I-labelled derivatives for ACTH and AII.

Ultrastructural studies using electron and light microscopy indicated that the cellular content of lipid droplets within the ZF/ZR cells increased following 3 days of culture. This may mean that cultured ZF/ZR cells contain higher levels of steroid precursors, able to contribute to the increase in basal steroidogenesis observed in the cultured cells. Crivello, Hornsby & Gill (1982) demonstrated a progressive deterioration of steroidogenesis with time in bovine ZG cell cultures; after several days of culture, aldosterone output was practically undetectable. To overcome this problem, they introduced metyrapone and the antioxidant dimethyl sulphoxide into the tissue culture medium. In contrast to the bovine ZG cell culture system, we observed an increase in cortisol secretion suggesting that, after primary culture, the metabolic state of the ZF/ZR cells has improved. It would appear that the enzymes responsible for cortisol synthesis are less susceptible to oxidation than those required for aldosterone biosynthesis.

No significant effect of noradrenaline was observed in freshly isolated purified ZF/ZR cells on cAMP or cortisol production over the concentration range 0·1 nmol–1 μmol noradrenaline/l. However, after culturing the ZF/ZR cells for 3 days, we observed a stimulatory effect of noradrenaline on cAMP and cortisol production, at a threshold concentration of 10 nmol/l for cortisol (Walker, Lightly, Milner & Williams, 1988). We have suggested that the lack of a significant effect of noradrenaline on steroidogenesis in freshly isolated ZF/ZR cells may be due to an in-vivo effect, such as stress, leading to desensitization of the β-receptors that slowly regain their sensitivity in culture as the in-vivo effect wears off.

In conclusion, comparison of the freshly isolated and cultured ZF/ZR cells from bovine adrenal cortex demonstrates clear differences in ultrastructural integrity and agonist responsiveness. The cultured cells display a significantly greater secretion of cortisol, both basal and in response to stimulation by ACTH and AII. They also respond steroidogenically to β-agonists, whereas the freshly isolated cells are completely unresponsive. In addition, the basal cAMP output and that in response to ACTH and β-agonists is also significantly greater after culture. These functional differences are reflected in better ultrastructural preservation of the cultured cells, which contain an increased density of lipid droplets and smooth endoplasmic reticulum.

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The technical advice and assistance of Miss E. Davies in the pilot experiments is gratefully acknowledged. We also wish to thank Dr F. A. O. Mendelsohn for providing the aldosterone antibody and SAPU for supplying the antiserum for cortisol. We would like to thank Professor L. G. Whitby for his critical comments and Mrs E. Ward for typing the manuscript. The work was supported by a grant from the Sir Stanley and Lady Davidson Medical Research Fund awarded to S.W.W. The support of the Wellcome Trust is also gratefully acknowledged. E.R.T.L. holds a Faculty of Medicine Scholarship and I.M.B. a Faculty of Medicine Fellowship from the University of Edinburgh.

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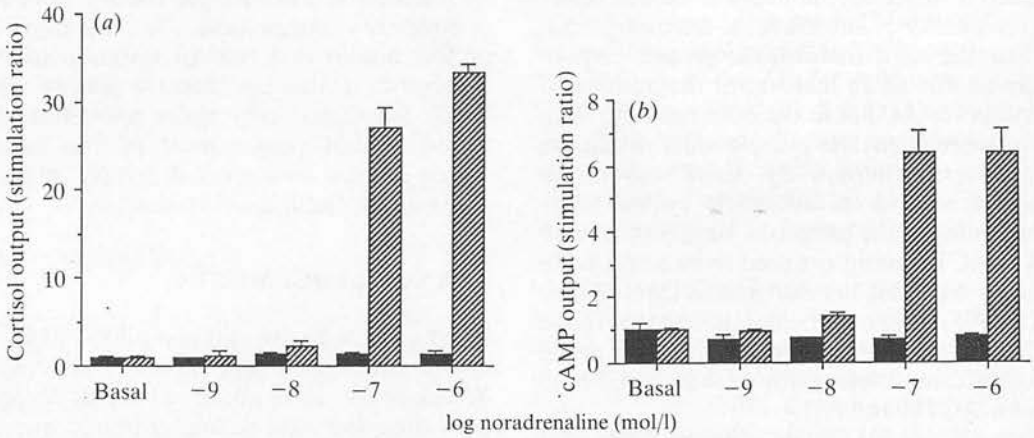

FIGURE 3. Output of (*a*) cortisol and (*b*) cAMP by bovine zona fasciculata/zona reticularis cells in response to noradrenaline. Cells were uncultured (solid bars) or cultured for 3 days (hatched bars), and results are expressed as a stimulation ratio relative to the basal (unstimulated) value on day 1 (uncultured) or day 3, as appropriate. Values are means ± S.E.M. for three wells.

(2–3 μm) stained with toluidine blue. In the freshly isolated cells, 4±1 lipid droplets per cell section ($n=100$ cell sections) were counted, compared with 25±2 droplets per cell section ($n=100$ cell sections) for the cells on day 3 of culture.

DISCUSSION

Characterization of the steroidogenic responsiveness and intracellular control mechanisms of cells isolated from the inner zones of the bovine adrenal cortex requires initial purification to remove cellular debris and red blood cells, and to minimize contamination by cells of the ZG. This is particularly important for studies involving ACTH and AII, since they act on both the ZG and ZF/ZR in the bovine species. Earlier studies on steroid secretion from bovine ZF/ZR cells did not include any purification step, and Hepp *et al.* (1977) reported that aldosterone, measured by a protein-binding assay, was still present in the final cell suspension.

In the present study, we employed the column filtration method originally devised for rat adrenal cell purification (McDougall *et al.* 1979) in order to isolate the ZF/ZR cells of the bovine adrenal cortex. Contamination by cells from the ZG was monitored using a sensitive and specific radioimmunoassay for aldosterone (Campbell *et al.* 1981). The aldosterone output from cells purified by column filtration was lower than the detection limit of the radioimmunoassay, suggesting that contamination by cells from the ZG is insignificant. No red blood cells or cellular debris could be seen under phase-contrast microscopy in the purified ZF/ZR cell preparation.

The comparisons between freshly isolated cells (day 1) and cultured cells on day 3 of culture was based on experiments that showed that the response to ACTH (0·1 nmol/l) was greatest on day 3. A similar result has been reported on an unpurified preparation of bovine adrenocortical cells (Kawamura, Nakamichi, Imagawa *et al.* 1984) and also in a partially purified preparation of bovine ZF/ZR cells (Duperray & Chambaz, 1980). Monolayer cultures of bovine ZF/ZR cells were also found by Goodyer, Torday, Smith & Giroud (1976) to show peak cortisol secretion within the first week of culture, thereafter declining. These findings contrast with those of O'Hare & Munro Neville (1973) using monolayer cultures of rat adrenocortical cells, where total steroid production (largely corticosterone) in response to ACTH remained high and relatively constant for 40 days or more in culture. In none of these studies has the agonist responsiveness of the freshly isolated (uncultured) cells been compared with the cultured cells, and the comparative effects of AII and catecholamines have never been examined. Neither is there any information on the effect of culture on the cAMP response to ACTH and catecholamines.

It could be argued that the differences between the response on day 3 of culture and the freshly isolated cells might be due to selective plating out of a responsive cell population, possibly cells least damaged by collagenase treatment. However, when dispersed into wells at 250 000 cells/ml, 80–90% of cells have attached to the plate after 24 h. Hence the cultured ZF/ZR population is representative of the freshly isolated ZF/ZR population.

Cyclic AMP production, both basal and in response to ACTH, was significantly increased after culture.

described for the freshly isolated purified ZF/ZR cells. At the end of the incubation period, the medium was carefully removed from the wells and assayed for cAMP, cortisol and, in some experiments, aldosterone by radioimmunoassay procedures. Following ethanol extraction, the cellular contents of cAMP were also measured.

Radioimmunoassay procedures

Cyclic AMP levels in the medium were determined by adding 0·5 ml of the medium to 2 ml absolute alcohol in a glass tube to precipitate protein. After centrifugation, the supernatant was dried and the extract reconstituted in 0·5 ml sodium acetate buffer (0·05 mol/l; pH 4·8) containing 0·1% BSA. Cellular levels of cAMP were measured by extracting the cells twice with 0·5 ml ethanol and reconstituting the dried-down extract in 0·5 ml sodium acetate buffer in the same way. Total cAMP was the sum of medium and cellular contents. Acetylation of the samples and the assay of cAMP were carried out according to the method of Harper & Brooker (1975). The interassay precision was less than 10% in the working range 0·30–3 nmol/l and less than 20% of the working range 0·1–20 nmol/l.

Cortisol output was measured in 100 µl of sample by direct radioimmunoassay (Gray, Seth & Beckett, 1983). The antibody for cortisol was kindly provided by the Scottish Antibody Production Unit, Carluke, Lanarkshire, U.K. The interassay precision was less than 10% over the working range 12–1000 nmol/l and less than 20% over the working range 5–12 nmol/l.

In the experiments where aldosterone outputs were measured, the antibody employed (402 L) was kindly provided by Dr F. A. O. Mendelsohn (Department of Medicine, The Austin Hospital, Melbourne, Australia). The characteristics of this antibody have been described (Campbell, Mendelsohn, Adam & Funder, 1981). The assay employed 50 µl of sample; it had an interassay coefficient of variation of less than 12% over the working range 0·2–20 nmol/l.

The standards used for the measurement of cAMP, cortisol and aldosterone were purchased from Sigma Chemical Co., Poole, Dorset, U.K.

Cellular protein content

This was measured on a minimum of three wells for each experiment, by adding 0·5 ml 1% (v/v) Triton X-100 (BDH, Glasgow, Strathclyde, U.K.) to each well. After diluting the sample 1 : 10 with water, protein content was determined by the method of Bradford (1976), automated for use on the Cobas Fara (Roche) centrifugal analyser. The assay standard was BSA made up in 0·1% Triton X-100.

Morphological analysis of cells

Cell pellets from days 1 and 3 of primary culture were fixed in 2·5% (v/v) glutaraldehyde in cacodylate buffer (0·1 mol/l, pH 7·3). Osmium tetroxide (1% in cacodylate buffer) was used as a secondary fixative. The fixed samples were dehydrated through graded alcohols into propylene oxide and finally embedded in Araldite resin. Thick sections (1 µm) were stained with toluidine blue for light microscopy. Ultrathin sections (50–60 nm) were stained with uranyl acetate and lead citrate for examination and photomicrography using a Philips EM300 electron microscope. Approximately 100 cells were viewed in at least three different ultrathin sections for each sample preparation.

Statistical analyses

Comparison of basal cortisol and cAMP values, and in response to agonist stimulation, employed Student's *t*-test for the freshly isolated purified ZF/ZR cells versus the same cells after primary culture. The comparison was carried out after normalizing the cortisol secretion rate with respect to cellular protein. When comparing the stimulated cortisol responses on days 1 (uncultured) and 3 (cultured), the basal value was first substracted.

RESULTS

The secretion of cortisol under basal conditions and in response to ACTH (1 nmol/l), AII (10 nmol/l) and adrenaline (1 µmol/l) was measured for the purified ZF/ZR cells on day 1 (uncultured) and days 2, 3, 4 and 5 (in culture). The basal and stimulated secretion of cortisol increased progressively to reach a peak on day 3, thereafter declining. The basal cortisol secretion and that in response to ACTH (1 nmol/l) on day 1 were $27 \pm 0{\cdot}5$ (S.E.M.) and $441 \pm 4{\cdot}8$ pmol/h per mg protein respectively. By day 3, the corresponding values had risen to $100 \pm 4{\cdot}5$ and $1542 \pm 17{\cdot}6$ pmol/h per mg protein respectively. By day 5, the basal secretion had declined to $80 \pm 3{\cdot}8$ and the secretion stimulated by ACTH (1 nmol/l) had fallen to $195 \pm 28{\cdot}9$ pmol/h per mg protein. All these values are for n = three wells. Detailed comparisons of cortisol production on the freshly isolated cells on day 1, and the same cell isolates on day 3 of primary culture were therefore carried out. In these experiments, the use of two steps in the wash-out procedure (see Materials and Methods) was found to lower basal values and lead to significant differences in n-fold stimulation as well as absolute cortisol secretion rates.

When the basal cortisol output was compared in this way, the normalized secretion rate was significantly ($P < 0{\cdot}05$) greater on day 3 than day 1 in six out of seven experiments. The stimulated cortisol output

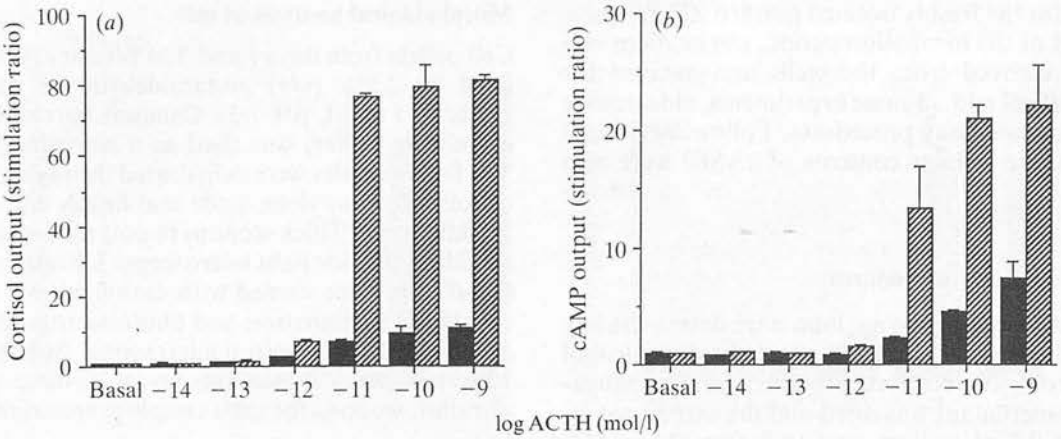

FIGURE 1. Output of (*a*) cortisol and *b*) cyclic (c)AMP by bovine zona fasciculata/zona reticularis cells in response to ACTH. Cells were uncultured (solid bars) or cultured for 3 days (hatched bars), and results are expressed as a stimulation ratio relative to the basal (unstimulated) value on day 1 (uncultured) or day 3, as appropriate. Values are means ± S.E.M. for three wells.

(normalized) was also significantly greater on day 3 for ACTH, AII and noradrenaline. Thus ACTH at 1 nmol/l achieved a greater cortisol output on day 3 in all six experiments where this comparison was made ($P<0{\cdot}01$ for six experiments). Likewise, AII at 10 nmol/l showed a very similar pattern, with cortisol secretion greater on day 3 in all experiments where this was examined ($P<0{\cdot}01$ for five experiments). In the case of noradrenaline, there was no significant stimulation of cortisol secretion at any dose used in the freshly isolated cells, whereas a highly significant stimulation was observed in culture on day 3 ($P<0{\cdot}001$ for all four experiments). The comparison for days 1 and 3 for a full dose–response of the agonists is illustrated in Figs 1 (ACTH), 2 (AII) and 3 (noradrenaline). The results are expressed as stimulation ratios (stimulated output/basal output) to enable the steroidogenic responsiveness of the cells to the three agonists to be more readily compared. No aldosterone was detectable in response to ACTH or AII using the column-purified ZF/ZR cells, either freshly isolated or in culture.

These differences might simply reflect deficient availability of substrate for steroidogenesis in the freshly isolated cells. To exclude this possibility, cAMP was measured in the experiment illustrated in Figs 1 and 3. The basal cAMP output was $1{\cdot}89 \pm 0{\cdot}17$ pmol/h per mg protein (n = five wells) on day 1, rising to $4{\cdot}52 \pm 0{\cdot}19$ pmol/h per mg protein by day 3 (n = six wells). The higher output on day 3 is statistically significant ($P<0{\cdot}001$). As with the cortisol response, the cells on day 1 showed no increase in cAMP production in response to any concentration of noradrenaline, whereas a dose-dependent rise occurred on day 3 of culture. For ACTH at 1 nmol/l, the total cAMP output was also higher on day 3

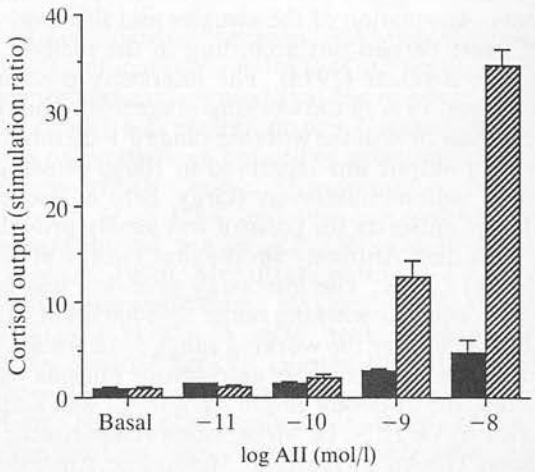

FIGURE 2. Cortisol response to angiotensin II (AII) for bovine zona fasciculata/reticularis cells on day 1 (uncultured) (solid bars) and on day 3 (cultured) (hatched bars), expressed as a stimulation ratio relative to the basal (unstimulated) value on day 1 (uncultured) or day 3, as appropriate. Values are means ± S.E.M. for three wells.

($P<0{\cdot}05$; n = three wells). No stimulation of cAMP production by AII was observed on either day.

An ultrastructural comparison of the cells on days 1 and 3 was also carried out. For all intact cells from these preparations, clear morphological differences were observed. The cultured cells consistently showed a higher density of smooth endoplasmic reticulum and lipid droplets (Plate).

To evaluate the extent of the difference in lipid content between freshly isolated cells and cultured cells (day 3), the number of lipid droplets per cell section was counted by light microscopy in thick sections

DESCRIPTION OF PLATE

An ultrastructural comparison of freshly isolated bovine zona fasciculata/zona reticularis (ZF/ZR) preparations on day 1 (uncultured) and day 3 (cultured). Scale bars are shown in μm.

FIGURE 1. A typical cell found in the freshly isolated bovine ZF/ZR preparation on day 1 (uncultured). N, nucleus; M, mitochondrion. 85–90% of the cells showed these features.

FIGURE 2. A cell found in the freshly isolated bovine ZF/ZR preparation on day 1 (uncultured). This cell shows poor integrity of the ultrastructure and an indistinct plasma membrane. A small percentage (10–15%) of the freshly isolated cells showed this appearance, but were not evident in the day 3 (cultured) preparation.

FIGURE 3. A representative cell found on day 3 of culture of the bovine ZF/ZR preparation. Numerous lipid droplets (L) are present, and the cell shows good integrity of ultrastructure.

FIGURE 4. A portion of the cell shown in fig. 3 showing good integrity of the plasma membrane and an abundance of smooth endoplasmic reticulum (ER).

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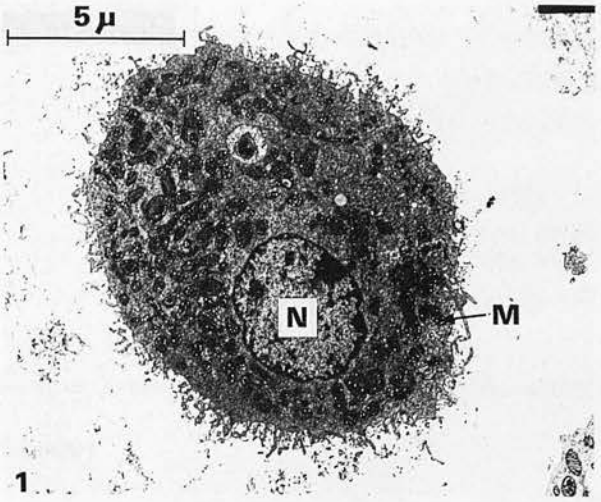
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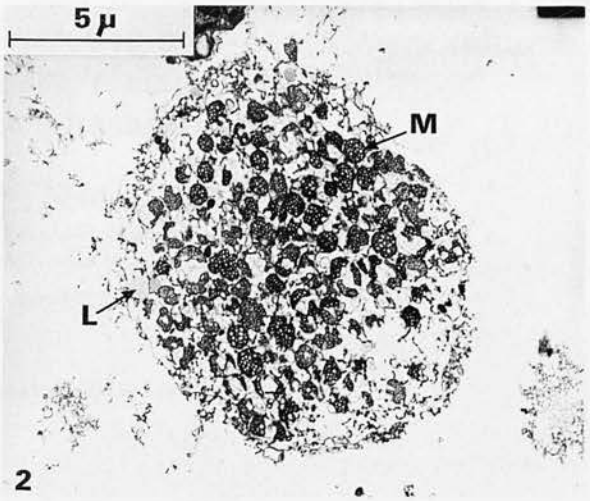
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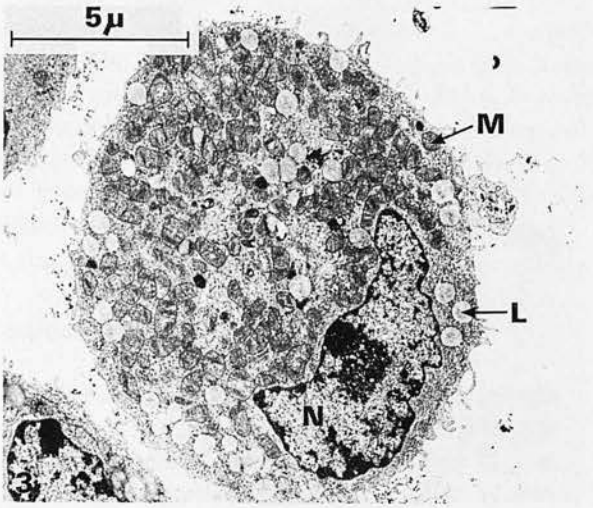
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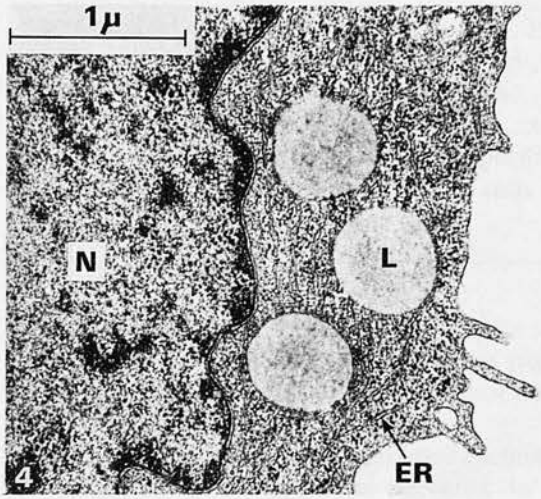
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Catecholamine stimulation of cortisol secretion by 3-day primary cultures of purified zona fasciculata/reticularis cells isolated from bovine adrenal cortex

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Key words: Adrenal cortex, cell culture; Cortisol; Cyclic AMP; Catecholamine; (Bovine)

Summary

Primary cultures of zona fasciculata/reticularis cells derived from bovine adrenal cortex secreted cortisol and corticosterone in response to isoprenaline, noradrenaline and adrenaline on the third day of culture. The potency order was isoprenaline > noradrenaline, adrenaline with an ED_{50} for all three agonists within the range $1–5 \times 10^{-8}$ M. A dose-dependent increase in medium content of cyclic AMP was also observed. Secretion of cortisol in response to these catecholamines was specifically blocked by propranolol but unaffected by phentolamine. The β-agonist effect on cortisol secretion was specifically and progressively reduced, in a time- and dose-dependent manner, by pre-incubation of the cells with adrenaline.

Introduction

ACTH is recognised as the major determinant of glucocorticoid secretion from the adrenal cortex, but other agonists such as angiotension II also stimulate glucocorticoid secretion in some species (Gill et al., 1977). An effect of catecholamines on adrenocortical function was suggested by Vogt (1944), who found that intravenous infusion of adrenaline or splanchnic nerve stimulation in dogs led to secretion of a factor(s) which prolonged the median survival of adrenalectomised rats. Subsequent work on hypophysectomised animals supported an effect of catecholamines on ACTH secretion rather than a direct effect on the adrenal cortex (Pickford and Vogt, 1951), but Inaba et al. (1975) reported a significant increase in plasma [corticosterone] following administration of adrenaline to hypophysectomised rats.

Stimulation of membrane adenylate cyclase activity by catecholamines was reported in the corticosterone-secreting tumour 494 from the rat (Schor et al., 1971; Brush et al., 1974). In humans, β-adrenergic receptors linked to adenylate cyclase were described in four out of six adrenocortical carcinomas, but not in three preparations derived from normal adrenal cortex (Katz et al., 1985).

Specific binding of the radioligand [^{3}H]dihydroalprenolol, a β-receptor antagonist, was demonstrated in two out of three cortisol-secreting adenomas of human origin (Hirata et al., 1981). Significant stimulation of aldosterone and corticosterone secretion by isoprenaline from the capsular fraction obtained from normal rat adrenal

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cortex was obtained by Shima et al. (1984); although the subcapsular fraction was apparently unresponsive, specific binding sites for [^{3}H]dihydroalprenolol were identified.

Using capsular explants of rat adrenal cortex in a perifusion system, Pratt et al. (1985) observed a stimulation of aldosterone secretion by β-agonists. De Lean et al. (1984) showed that β-agonists would stimulate aldosterone secretion from 3-day primary cultures of bovine adrenocortical cells. Kawamura et al. (1984) also examined the effect of catecholamines on steroidogenesis from primary bovine adrenal cultures on the second day of culture and found a dose-dependent increase in steroid output accompanied by a rise in cyclic AMP; however, no attempt was made in this study to separate the zona fasciculata/reticularis cells from the zona glomerulosa cells and steroid secretion was quantitated by a non-specific fluorimetric method.

We have recently established and optimised a primary culture system for purified bovine zona fasciculata/reticularis cells. This system is highly responsive to ACTH, and has been used to clarify the possible effect of catecholamines on glucocorticoid secretion from zona fasciculata cells. In addition to eliminating zona glomerulosa cells from the cell preparation by column purification, we have also employed specific radioimmunoassay methods for determining the secretory responses of the two major glucocorticoids, cortisol and corticosterone.

Materials and methods

Materials

Bovine adrenal glands were obtained from freshly slaughtered cattle at the Edinburgh District Council slaughterhouse. Ham's F12 growth medium, fetal calf serum, Earle's balanced salt solution, glutamine and cell culture plastics were obtained from NBL, Cramlington, U.K. Penicillin, streptomycin and fungizone were from Flow Laboratories, Rickmansworth, U.K. Collagenase was from Lorne Diagnostics, Bury St. Edmunds, U.K. and bovine serum albumin (fraction V) from ICN Biomedical, High Wycombe, U.K. Sephadex G-10 and G-50 were from Pharmacia, Milton Keynes, U.K. The sources of the agonists and antagonists were as follows: isoprenaline (0.1 mg/ml) from McCarthay's, Romford, U.K.; adrenaline (1 mg/ml) from Phoenix Pharmaceuticals, Gloucester, U.K.; noradrenaline (1 mg/ml) from Winthrop Laboratories, Horsham, U.K.; propranolol from Imperial Chemical Industries, Macclesfield, U.K.; Synacthen from CIBA Laboratories, Horsham, U.K.

Cortisol, corticosterone and adenosine 3′,5′-cyclic monophosphate (cyclic AMP) standards were obtained from Sigma Chemical Company, Poole, U.K. Radioisotopes for the cyclic AMP and corticosterone assays were synthesised by the methods of Harper and Brooker (1975) and Al-Dujaili et al. (1981), respectively. The cortisol label, cortisol-3-(*O*-carboxymethyl) oximino-(2-[^{125}I]iodohistamine), and [^{125}I]NaI were from Amersham International, Aylesbury, U.K.

The cortisol antiserum was obtained from the Scottish Antibody Production Unit, Carluke, U.K.

Methods

Cell culture. Primary cultures of bovine adrenocortical cells were established as follows. Adrenal glands from freshly slaughtered animals were collected on ice into phosphate-buffered saline and transported within 20 min to the laboratory. After the surrounding fat was carefully removed, the cortex was sectioned into 100 μM slices with a Stadie-Riggs microtome; the first slice, which (by light microscopy) can be shown to consist of the capsule and underlying zona glomerulosa, was discarded. The second slice, consisting of zona fasciculata/reticularis tissue, was collected into Earle's balanced salt solution containing 0.2% (w/v) bovine serum albumin (BSA) (EBS/0.2% BSA).

After mincing the zona fasciculata/reticularis with scissors, the tissue was digested for 1.5 h in EBS containing 2% (w/v) BSA with collagenase added to a final concentration of 1.5–2 mg/ml. The tissue was mechanically disrupted by repeated pipetting at 30 min, 60 min and 90 min during the incubation. At the end of the collagenase digestion, undigested tissue was removed through 250 μM nylon gauze and the cells harvested by centrifugation at $300 \times g$ (4° C). After resuspension in EBS/0.2% BSA, the cells were filtered through 100 μM and then 30 μM gauze and

finally applied to a column containing 5 ml of Sephadex G-10 in order to trap the intact cells amongst the Sephadex beads. The Sephadex G-10 was overlaid by 5 ml of Sephadex G-50 (coarse) to act as a pre-filter.

The column was washed with EBS/0.2% BSA, and the cells then harvested by passing the resuspended gel through 30 μM gauze; this traps the Sephadex beads but allows the cells through. Cells prepared in this way were substantially free from debris, red blood cells and clumps of cells.

The cells were counted and then plated out in 12-well plates in Ham's F12 medium containing 10% (v/v) fetal calf serum with added penicillin (50 IU/ml), streptomycin (50 μg/ml) and amphotericin B (2.5 μg/ml), with 2.5×10^5 cells per well. Culture was carried out at 37°C in an atmosphere of 5% CO_2. The medium was changed on the second day and the agonist experiments set up on the third day.

Cultured cells are characterised by a threshold response to ACTH of 10^{-12} M and a peak response to 10^{-10} M ACTH, with a 20- to 30-fold stimulation (relative to basal) at the peak ACTH concentration (third day).

Agonist / antagonist experiments. All experiments were carried out on the third day of culture unless otherwise stated. The medium was removed and the cells washed once with EBS/0.2% BSA.

Agonists and antagonists were made up freshly in EBS/0.2% BSA with 0.1% (w/v) added glucose (EBS/0.2% BSA/0.1% glucose) to the concentration shown in the Results section; they were added (as 1 ml) to three wells for each concentration. In all experiments, the basal cortisol output was determined in 1 ml of EBS/0.2% BSA/0.1% glucose alone.

Incubations were for 1–2 h in 5% CO_2 at 37°C and were terminated by transferring the overlying solution to tubes cooled on ice. After centrifugation for 10 min at $300 \times g$ (4°C), 250 μl of cell-free supernatant was added to 1 ml of ice-cold ethanol, for the cyclic AMP assay; the remaining 750 μl was transferred to a fresh tube, for the corticosterone and cortisol assays. Samples were stored prior to assay at −20°C; the thawed samples were centrifuged immediately before assay to remove protein precipitated on storage. All experiments were repeated at least three times.

In some experiments, adrenaline or ACTH was present in the growth medium either throughout the whole period of culture or for shorter, defined time-periods before setting-up the agonist experiments on the third day. In these experiments, the wells were washed twice with EBS/0.2% BSA/0.1% glucose before exposure to the agonist.

To standardise the cortisol, corticosterone and cyclic AMP secretion rates, results were expressed as pmol per h per 10^5 cells, with cells counted in all three wells. In later experiments, it was found more convenient to standardise secretion rates relative to the protein content in the well. Protein was measured after digesting the cells in 1% Triton-X, using the method of Bradford (1976), adapted for use on the Cobas Fara (Roche) centrifugal analyser. Samples were diluted 1 : 10 with water before the Bradford assay and bovine serum albumin standards made up in 0.1% Triton-X. Higher concentrations of Triton-X were found to increase the absorbance at the monitoring wavelength of 595 nm.

Assays for cortisol, corticosterone and cyclic AMP. Cyclic AMP, cortisol and corticosterone were measured by in-house radioimmunoassays.

The cyclic AMP assay was carried out essentially as described by Harper et al. (1975). Ethanol extracts were dried down at room temperature under a stream of nitrogen and reconstituted in EBS prior to acetylation. Standards were also prepared and acetylated in EBS. The first antibody incubation was overnight at 4°C, with a charcoal separation for the second stage.

The corticosterone assay used an overnight incubation with first antibody at 4°C and a charcoal separation for the second stage (Al-Dujaili et al., 1981).

The cortisol assay used a single incubation for 70 min with an antibody pre-precipitate as described by Gray et al. (1983).

The between-assay CV was 10% or less over the working range for the cortisol and corticosterone assays and 13% or less over the working range for the cyclic AMP assay.

Results

The effect of catecholamines on cortisol and corticosterone secretion

The effects of isoprenaline, noradrenaline and

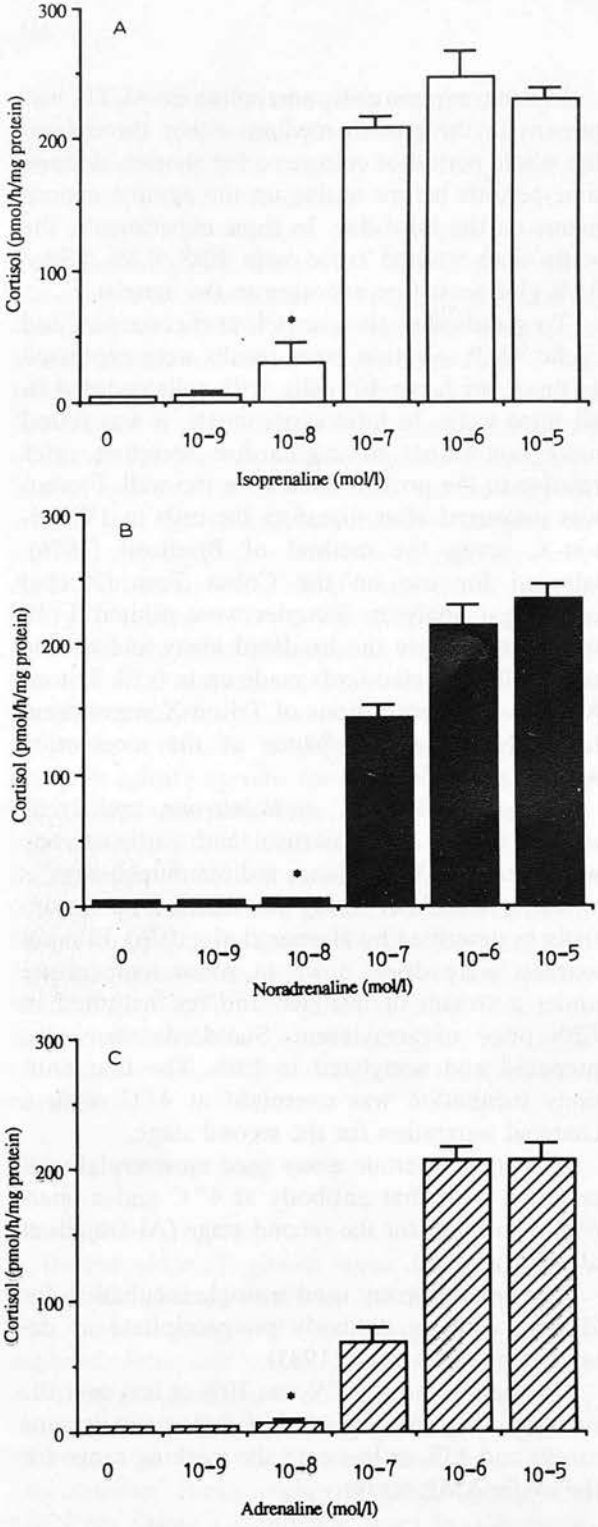

Fig. 1. An illustrative experiment showing the effect of isoprenaline, noradrenaline and adrenaline on the secretion of cortisol from 3-day primary cultures of zona fasciculata/reticularis cells. Each bar represents the mean cortisol secretion rate $\pm$SD ($n = 3$ wells) for the concentration of agonist used. The threshold concentration of agonist producing a significant response ($P < 0.05$) relative to basal secretion (0; no agonist) is shown (*).

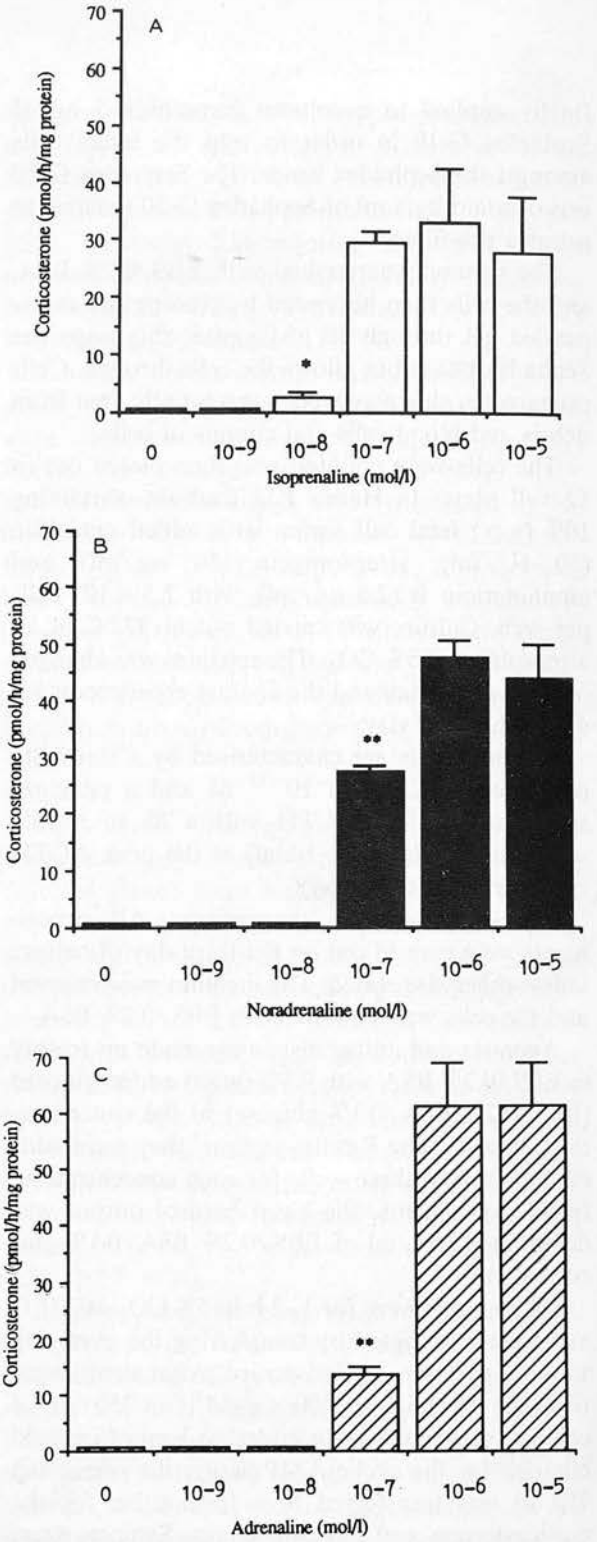

Fig. 2. An illustrative experiment showing the effect of isoprenaline, noradrenaline and adrenaline on the secretion of corticosterone from 3-day primary cultures of zona fasciculata/reticularis cells. Each bar represents the mean corticosterone secretion rate $\pm$SD ($n = 3$ wells) for the agonist concentration used. The threshold concentration of agonist producing a significant response ($P < 0.05$) relative to the basal secretion (0; no agonist) is shown (*).

TABLE 1

THE EFFECTS OF CATECHOLAMINES ON CORTISOL SECRETED FROM CULTURED ZF CELLS

Agonist	Number of experiments	Mean ED_{50} (M)	Range (M)
Isoprenaline	3	1.3×10^{-8}	$0.3–5.0\times10^{-8}$
Noradrenaline	5	3.2×10^{-8}	$1.3–7.9\times10^{-8}$
Adrenaline	3	5.0×10^{-8}	$2.0–12.6\times10^{-8}$

adrenaline on the output of cortisol and corticosterone from cultured zona fasciculata/reticularis cells on the third day of primary culture are shown in Figs. 1 and 2, respectively. All three agonists produced a dose-dependent increase in cortisol and corticosterone secretion compared with basal secretion over the same period. The potency order was isoprenaline > noradrenaline, adrenaline. A summary of the effects of the three agonists on cortisol secretions is shown in Table 1.

The cyclic AMP response to catecholamines

The threshold catecholamine concentration producing a significant increase in cyclic AMP production occurred at 10^{-8} M for isoprenaline and 10^{-7} M for noradrenaline and adrenaline. A dose-dependent increase in cyclic AMP was observed with all three β-agonists (Fig. 3).

The effects of α- and β-blockade

To establish the nature of the catecholamine receptor responsible for the stimulation of cortisol secretion, the effects of α-blockade (with phentolamine) and β-blockade (with propranolol) were examined. Phentolamine (10^{-6} M) had no effect on the cortisol secretory response achieved by 10^{-7} M isoprenaline, noradrenaline or adrenaline (Fig. 4), whereas 10^{-6} M propranolol abolished

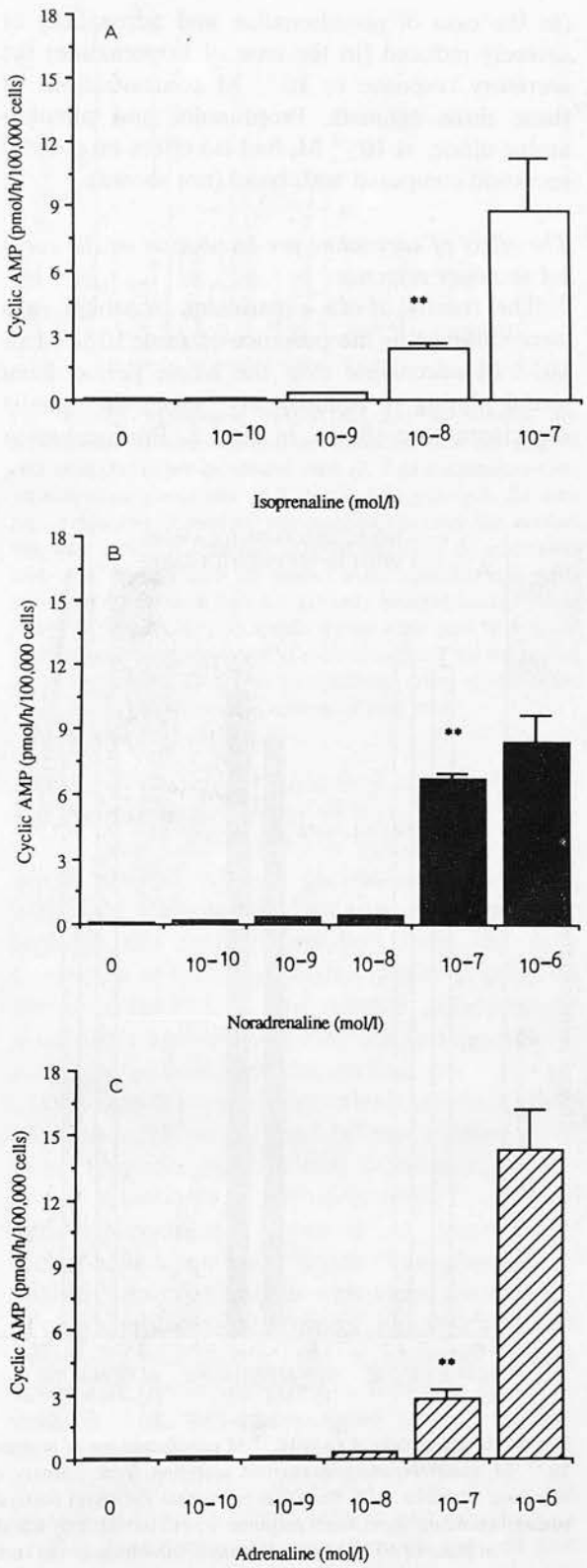

Fig. 3. An illustrative experiment showing the effect of isoprenaline, noradrenaline and adrenaline on cyclic AMP production from 3-day primary cultures of zona fasciculata/reticularis cells. Significant stimulation relative to basal cyclic AMP production (0; no agonist) at a level $P < 0.01$ is shown (**). Each bar represents the mean cyclic AMP production $\pm$SD ($n = 3$ wells) for the agonist concentration used, showing the SD only where these values were sufficiently large to appear on the scale used.

(in the case of noradrenaline and adrenaline) or severely reduced (in the case of isoprenaline) the secretory response to 10^{-7} M concentrations of these three agonists. Propranolol and phentolamine alone, at 10^{-6} M, had no effect on cortisol secretion compared with basal (not shown).

The effect of adrenaline pre-incubation on the cortisol secretory response

The results of an experiment in which cells were cultured in the presence of zero, 10^{-7} M or 10^{-6} M adrenaline over the whole period from initial plating to immediately before the agonist experiment are shown in Fig. 5. Pre-incubation with 10^{-7} M adrenaline significantly reduced, and pre-incubation with 10^{-6} M adrenaline completely abolished, the cortisol response to re-challenge with adrenaline.

Parallel experiments, in which cells were pre-incubated with 10^{-11} M ACTH over the same period, showed that ACTH and adrenaline responses on the third day were not significantly different from the control response.

The time-course for the decrease in cortisol secretory response produced by pre-incubation with adrenaline (10^{-7} M) shows that the cortisol response is significantly impaired after 2 h and reduced by 84% at 4 h (Fig. 6). Cyclic AMP

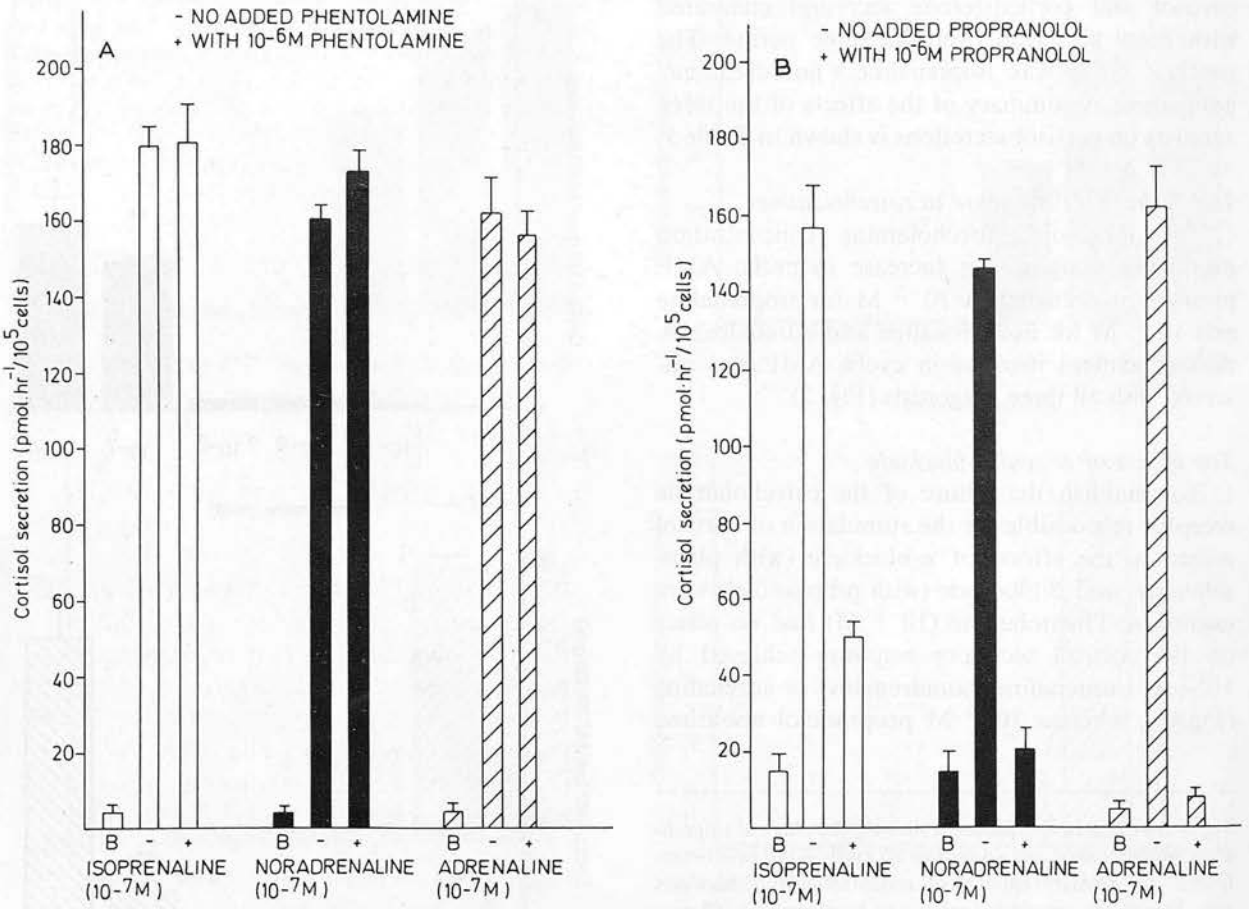

Fig. 4. The effect (Fig. 4*A*) of 10^{-7} M concentrations of isoprenaline, noradrenaline and adrenaline, with and without the addition of 10^{-6} M phentolamine, on cortisol secretion from primary cultures (third day) of zone fasciculata/reticularis cells. The basal secretion is shown at B. Each bar represents the mean cortisol secretion rate $\pm$SD ($n = 3$ wells). No significant effect of 10^{-6} M phentolamine on the cortisol response was observed. Fig. 4*B* shows the same experiment, except that 10^{-6} M propranolol was used in place of 10^{-6} M phentolamine. Inhibition of the cortisol response to 10^{-7} M agonist by propranalol was observed.

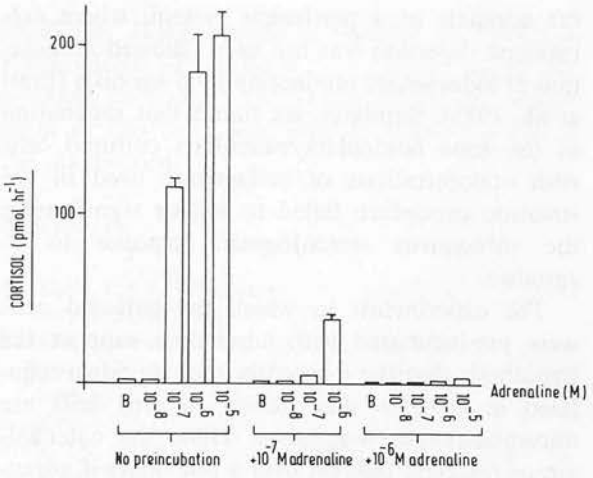

Fig. 5. The effects of pre-incubating cultured zona fasciculata/reticularis cells with adrenaline on the subsequent cortisol dose-response to adrenaline on day 3 of culture. An illustrative experiment is shown. Cells were plated out at 2.5×10^5 cells power well with zero, 10^{-7} M or 10^{-6} M adrenaline; this was replenished on the second day and washed out before carrying out the full dose-response experiments with adrenaline on the third day. Each bar represents the mean cortisol response $\pm$SD ($n = 3$ wells), showing the SD only where this is sufficiently large to appear on the scale used. The highly significant decline in cortisol response achieved by pre-incubation with 10^{-7} M adrenaline, and the abolition of response by 10^{-6} M adrenaline are evident. Adrenaline pre-incubation had no significant effect on the protein content of the wells as compared to the control (no adrenaline) pre-incubation.

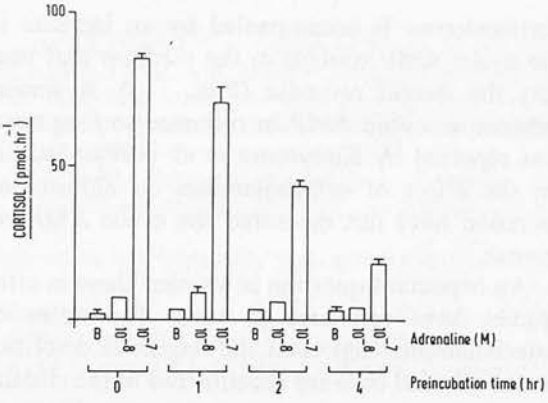

Fig. 6. An experiment illustrating the time-course for desensitisation of the response to adrenaline when the cultured cells (day 3) are pre-incubated with 10^{-7} M adrenaline. Cells were initially plated out at 2.5×10^5 cells per well. At each time-point, the adrenaline was washed out and the cortisol response to zero (i.e. basal), 10^{-8} M and 10^{-7} M adrenaline measured. Results show the mean cortisol secretion rate $\pm$SD ($n = 3$ wells) for each bar, the SD only being indicated where this is sufficiently large to appear on the scale used. A progressive decline in cortisol secretion is observed over the 4 h period of the experiment. There was no significant effect of adrenaline on the protein content of each well.

output was also measured in some experiments. There was a similar decline (not shown) in the responsiveness to adrenaline re-challenge following pre-incubation with adrenaline.

Discussion

Several previous studies examined the effects of catecholamines on aldosterone production in both rat and bovine species (De Lean et al., 1984; Pratt et al., 1985; Sequeira and McKenna, 1985). However, there is much less information on the effect of catecholamines on zona fasciculata function. De Lean et al. (1984) reported that β-agonists had no effect on cortisol secretion from bovine zona fasciculata cells, but did not state whether freshly isolated cells or cultured cells were used in these experiments. Although Kawamura et al. (1984) reported secretion of steroids from 2-day primary cultures of bovine adrenocortical cells, their study was limited in a number of ways. Total steroid output was measured by a non-specific fluorimetric method, without specific measurement of individual hormones, and no attempt was made to separate the zona glomerulosa from the zona fasciculata to define a specific β-agonist effect on the zona fasciculata. The marked differences in β-agonist responsiveness between freshly isolated and cultured cells were not studied.

Our results support and extend the findings of previous work on cultured bovine adrenocortical cells. They demonstrate that catecholamines are potent stimulators of steroidogenesis. The cortisol and corticosterone responses to isoprenaline, noradrenaline and adrenaline have been consistently observed in experiments on the cultured bovine zona fasciculata/reticularis cells. The most potent catecholamine was isoprenaline which showed a threshold response between 10^{-10} M and 10^{-9} M; this hierarchy of potency is consistent with the specific inhibition of the catecholamine effect by the β-blocker, propranolol. The α-blocker, phentolamine had no effect.

The dose-dependent secretion of cortisol and

corticosterone is accompanied by an increase in the cyclic AMP content in the medium that mirrors the steroid response (Figs. 1–3). A similar increase in cyclic AMP in response to β-agonists was reported by Kawamura et al. (1984). Studies on the effect of catecholamines on aldosterone secretion have not measured the cyclic AMP response.

An important question is whether these in vitro studies have relevance in vivo. The doses of catecholamines that elicit the responses described in the cultured cells are encountered in the circulation during the stress response in man (Barrand and Callingham, 1983). Furthermore, there is increasing evidence for an adrenergic innervation of the adrenal cortex in some mammalian species (Kleitman and Holzwarth, 1985; Edwards and Jones, 1987). In the hypophysectomised rat, infusion of isoprenaline produces a significant elevation in plasma [corticosterone] (Inaba and Kamata, 1975), and adenylate cyclase activity linked to β-agonist stimulation has been reported in adrenocortical tissue, both normal and neoplastic (Schor et al., 1971; Brush et al., 1974; Hirata et al., 1981; Shima et al., 1984; Katz et al., 1985).

In our experience, and also that of Kawamura et al. (1984), cells freshly isolated from the adrenal cortex show no response to catecholamines. Sequeira and McKenna (1985) also reported that adrenaline and noradrenaline were without effect on aldosterone production from freshly isolated suspensions of bovine zona glomerulosa cells. These findings suggest that the β-receptor is absent in vivo and is acquired in culture, as has been described for cultured hepatocytes (Nakamura et al., 1983). However, several other explanations are possible: the β-receptor may be damaged during the cell isolation procedure, or the β-receptor is present but shows a high β-agonist occupancy, or the β-receptor is down-regulated in vivo as a result of high circulating catecholamine levels, β-adrenergic nerve activity or a combination of both.

We have found that the cultured zona fasciculata cells are far more responsive to agonists such as ACTH compared with freshly isolated cells. Loss by degradation of the β-receptor during the cell isolation period, with recovery during culture, cannot be ruled out. Against this possibility, however, experiments on capsular explants from rat adrenals in a perifusion system, where collagenase digestion was not used, showed stimulation of aldosterone production by β-agonists (Pratt et al., 1985). Similarly, we found that incubation of the zona fasciculata/reticularis cultured cells with concentrations of collagenase used in the isolation procedure failed to impair significantly the subsequent steroidogenic response to β-agonists.

The experiments in which the cultured cells were pre-incubated with adrenaline support the hypothesis that the β-receptor may be down-regulated in vivo, so that freshly isolated cells are unresponsive to β-agonists. Thus, the catecholamine response falls off over a few hours if adrenaline is present in the growth medium, falling to less than 20% of the response seen normally with 10^{-7} M adrenaline after as little as 4 h pre-incubation. The phenomenon is not related to substrate exhaustion since the ACTH response is unaffected. This observation resembles the loss in aldosterone secretion in response to β-agonist stimulation observed in co-cultures of bovine adrenocortical and adrenomedullary cells (De Lean and Racz, 1985).

It is possible that the β-receptor is important in the cortisol response to acute stress, whereas in situations of more chronic stress the predominant stimulator of cortisol secretion is ACTH. With a stress response lasting more than a few hours, down-regulation of the β-receptor would occur, but ACTH levels would remain elevated to maintain the steroid response. In view of the stressful circumstances that obtain for some hours before removal of adrenal glands from animals at a slaughterhouse, this hypothesis could account for the absent β-response in the freshly isolated cells.

Our results support the possibility that catecholamines may regulate steroidogenesis in vivo. They provide another example of the important interactions which exist between the adrenal cortex and medulla (Weinkove and Anderson, 1986).

Acknowledgements

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Subclassification of β-adrenoceptors responsible for steroidogenesis in primary cultures of bovine adrenocortical zona fasciculata/reticularis cells

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1 Forty eight hour primary cultures of purified bovine adrenocortical zona fasciculata/reticularis cells secreted hydrocortisone in response to stimulation with β-adrenoceptor agonists. The observed order of potency was isoprenaline > noradrenaline > dobutamine > salbutamol > BRL37344.

2 Salbutamol acted as a partial agonist on these cells hence suggesting the presence of a β_1-adrenoceptor.

3 Schild analysis of the hydrocortisone response to isoprenaline showed that the selective β_1-antagonist practolol and the selective β_2-antagonist ICI118,551 gave pA_2 values of 6.85 and 7.17, respectively. These values were in close agreement with corresponding pA_2 values previously obtained for the β_1-adrenoceptor.

4 We conclude that β_1-adrenoceptors are responsible for mediating catecholamine-stimulated hydrocortisone secretion from primary cultures of bovine zona fasciculata/reticularis cells.

Introduction

Adrenocorticotrophic hormone (ACTH) and, in some species, angiotensin II are known regulators of steroidogenesis in the adrenal cortex (Gill *et al.*, 1977; Tait *et al.*, 1980). However, there is also evidence for innervation of the adrenal cortex in man (Mikhail & Amin, 1969), the rat (Kleitman & Holzwarth, 1985) and the mouse (Migally, 1979), and this has been suggested as another means of controlling adrenocortical steroidogenesis and possibly adrenal cell growth.

We have previously shown that primary cultures of bovine zona fasciculata/reticularis (ZFR) cells produce hydrocortisone in response to stimulation with catecholamines, and that this adrenergic stimulation of steroidogenesis is mediated by β-adrenoceptors (Walker *et al.*, 1988). The subclass of β-adrenoceptors which is involved in this response—either β_1 or β_2 as classified by Furchgott (1972), or the more recently characterised β_3 subclass (Arch *et al.*, 1984; Kaumann, 1989)—is unknown.

It is not yet clear in bovine or other species whether adrenergic control of hydrocortisone secretion at the level of the adrenal cortex occurs *in vivo* and, if so, whether this depends on adrenergic innervation, on the effects of circulating catecholamines, or even on catecholamines locally derived from the adrenal medulla. Ungar (1979) suggested that, in general, β_1-adrenoceptors tend to be innervated by adrenergic neurones, whereas β_2-adrenoceptors respond mainly to blood-borne catecholamines. On this basis, determination of the subclass of β-adrenoceptor should provide valuable evidence as to whether adrenergic control of the adrenal cortex is by direct innervation or via circulating catecholamines.

Traditional receptor classification is based on determination of antagonist pA_2 values (Schild, 1947; Kenakin, 1982), and comparison of a range of selective agonists (Lands *et al.*, 1967). Subclassification of β-adrenoceptors has been successfully demonstrated with the selective β_1-antagonist practolol (Dunlop & Shanks, 1968) and the selective β_2-antagonist ICI118,551 (Bilski *et al.*, 1983). These antagonists have been used in this present study. The effects of the selective β-agonists noradrenaline (β_1), salbutamol (β_2), dobutamine (β_1), isoprenaline (β_1/β_2), and BRL37344 (β_3) were also compared.

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Methods

Cell culture and stimulation

Bovine adrenal ZFR cells were prepared as described by Walker *et al.* (1988) using collgenase digestion, and purified by the column-filtration method developed by McDougall *et al.* (1979). This procedure gives a preparation essentially free from zona glomerulosa cell and medullary cell contamination (Williams *et al.*, 1989). The ZFR cells were dispersed into 12-well culture dishes (1.5 cm wells) at 250,000 cells per well in Ham's F10 medium containing 10% (v/v) CPSR5, penicillin (50 iu ml^{-1}), streptomycin (50 $\mu g\,ml^{-1}$) and amphotericin B (2.5 $\mu g\,ml^{-1}$), and cultured at 37°C under 5% CO_2. After 24 h (day 2), the medium was replaced with 1 ml of identical fresh medium.

Experiments were carried out 48 h after initial plating (day 3). Medium was removed and cells were washed twice with 1 ml of Earl's balanced salt (EBS) solution containing 0.2% bovine serum albumin (BSA) and 0.1% added glucose (EBS/BSA/glucose). Agonists and antagonists were made up in EBS/BSA/glucose and added to the cells, giving a final volume of 1 ml per well. Antagonists were added 1 min before the addition of any agonists. Stimulation was carried out for 1 h under the same incubation conditions as those used to culture the cells and, at the end of this period, the medium overlying the cells was removed and stored at −20°C before assay for hydrocortisone.

Hydrocortisone was measured by radioimmunoassay as described by Gray *et al.* (1983). The inter-assay CV was 10% or less over the working range of the assay.

Statistical analysis

Experiments were carried out on cells from at least 3 separate cell preparations for determination of agonist potencies and for Schild analysis of each antagonist. Within each experiment, triplicate determinations were carried out for each combination of agonist and antagonist.

For the estimation of antagonist pA_2 values, dose-response curves were tested for parallelism by analysis of covariance

using the SPSS-X statistical package produced by Edinburgh University Computing Service. Schild regression lines were fitted with a least-squares fit by a Casio fx-180P programmable calculator and confidence limits calculated.

Materials

The source of all cell culture and radioimmunoassay materials is described in Walker *et al.* (1988). The controlled process serum replacement No. 5 (CPSR5) was obtained from Sigma Chemical Company, Poole, Dorset, U.K.

Noradrenaline was obtained from Winthrop, Guildford, Surrey, U.K.; salbutamol from Allen & Hanburys Ltd., London, U.K.; dobutamine from Eli Lilly & Co. Ltd., Basingstoke, U.K. and isoprenaline from Macarthy Medical, Romford, U.K. BRL37344 ((**R*R***)-(±)-4-[2-[2-hydroxy-2-(3-chlorophenyl)-ethylamino]propyl]phenoxyacetic acid. Na salt) was a generous gift from Beecham Pharmaceuticals, Epsom, Surrey, U.K. Practolol was obtained from ICI plc, Macclesfield, Cheshire, U.K. and ICI 118,551 (erythro-(±)-1-(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol) was a generous gift from the same company.

Results

The effects of various selective β-agonists on hydrocortisone secretion

The effects of increasing concentrations of isoprenaline, noradrenaline, salbutamol, dobutamine and BRL37344 were tested on cells on day 3 of culture (Figure 1). The agonists had relative potencies as follows: isoprenaline > noradrenaline > dobutamine > salbutamol > BRL37344. Isoprenaline, noradrenaline and dobutamine all produced the same maximum response. Salbutamol and BRL37344 gave approximately 70% and 19% of the maximum response, respectively.

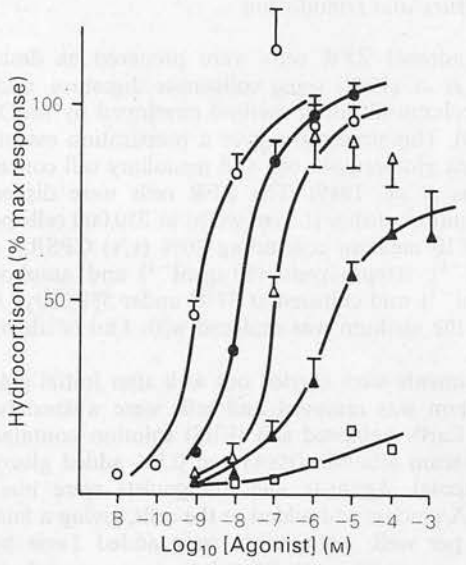

Figure 1 Dose-response curves for the secretion of hydrocortisone produced upon stimulation of purified bovine adrenocortical zona fasciculata/reticularis cells with isoprenaline (○), noradrenaline (●), dobutamine (△), salbutamol (▲) and BRL37344A (□) B = basal cortisol production.

The effects of the antagonists practolol and ICI118,551 on the isoprenaline dose-response curve

A series of dose-response curves for the effect of isoprenaline on hydrocortisone secretion were set up in the presence of increasing doses of either practolol or ICI118,551. Representative experiments for practolol and ICI118,551 are shown in Figures 2 and 3, respectively. The dose-response lines for each concentration of antagonist were judged to be parallel by analysis of covariance. Experiments were repeated on cells from 4 separate cell preparations for practolol and 3 separate cell preparations for ICI118,551.

The dose-ratio (DR) for each concentration of antagonist was obtained, Schild plots of $\log_{10}$ (DR − 1) versus antagonist

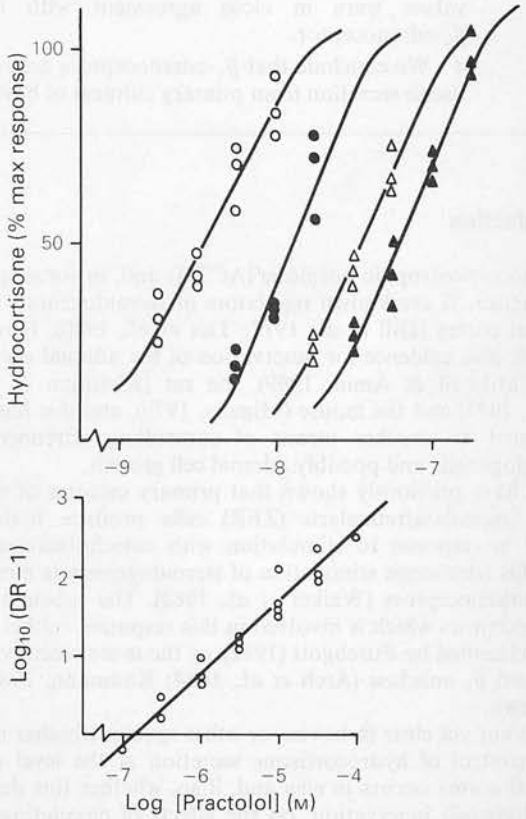

Figure 2 Representative experiment showing dose-response curves for the secretion of hydrocortisone produced on stimulation with isoprenaline, alone (○) and in the presence of increasing concentrations of practolol $10^{-6.5}$ M (●), 10^{-6} M (△), $10^{-5.5}$ M (▲). Inset: Schild regression—least squares fit of $\log_{10}$ (DR − 1) versus $\log_{10}$ [practolol] where DR = dose-ratio. Cumulative data from 4 separate cell preparations.

Table 1 Comparison of experimental and published data for practolol and ICI118,551

Antagonist	*Experimental* pA_2	*95% CL*	*Slope*	*95% CL*	*Published* pA_2
		6.67		0.84	
Practolol	6.85		0.90		6.80[1] (β_1)
		7.06		0.96	
		7.03		1.01	
ICI118,551	7.14		0.99		7.17[2] (β_1)
		7.28		0.97	9.26[2] (β_2)

95% CL = 95% confidence limits. Original references are [1]Kenakin & Black (1978), [2]Bilski *et al.* (1983).

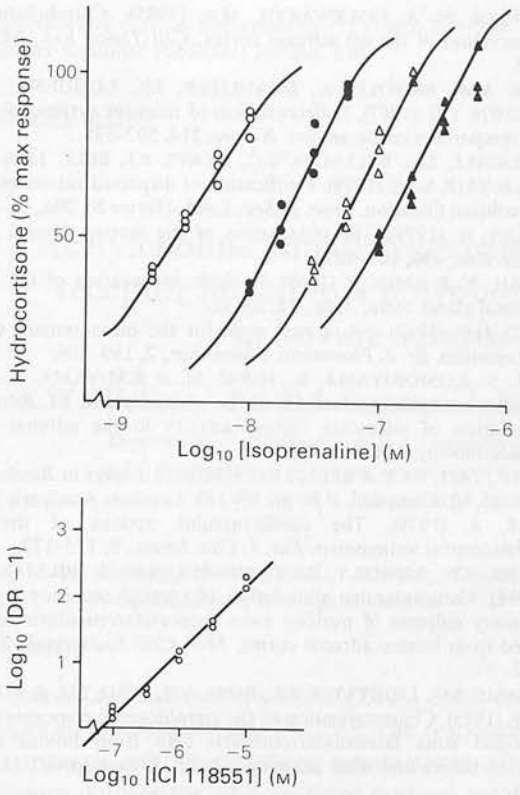

Figure 3 Representative experiment showing dose-response curves for the secretion of hydrocortisone produced on stimulation with isoprenaline, alonr (○) and in the presence of increasing concentrations of ICI118,551 $10^{-6.5}$ M (●), 10^{-6} M (△) $10^{-5.5}$ M (▲). Inset: Schild regression—least squares fit of $\log_{10}(DR - 1)$ versus $\log_{10}$[ICI118,551] where DR = dose-ratio. Cumulative data from 3 separate cell preparations.

concentration plotted (lower sections in Figures 2 and 3) and used to calculate pA_2 values for each antagonist and to obtain 95% confidence limits. The slope of the regression line and 95% confidence limits were estimated similarly. Results for both antagonists are shown in Table 1.

Neither practolol nor ICI118,551 caused hydrocortisone secretion and therefore had no intrinsic agonist effects on the cells.

Discussion

The results establish the existence of β_1-adrenoceptors on bovine cultured adrenal ZFR cells, for two reasons, as discussed below.

Firstly, the effects of several selective β-adrenoceptor agonists on hydrocortisone secretion were consistent with this classification. Isoprenaline, noradrenaline and dobutamine were all full agonists, whereas salbutamol acted as a partial agonist, producing 70% of the maximum response. Salbutamol is known to act as a full agonist at β_2-adrenoceptors, but only as a partial agonist at β_1-adrenoceptors (Farmer *et al.*, 1970). This, in itself, suggests that the adrenergic stimulation of hydrocortisone secretion is mediated by β_1-adrenoceptors. Although BRL37344 produced stimulation of the cells at 10^{-5} M, it was the least potent of all the agonists studied and only produced 19% of the maximum response seen with isoprenaline. Previous studies have shown that, in systems thought to contain β_3-adrenoceptors, BRL37344 was a more potent agonist than isoprenaline (Arch *et al.*, 1984; Bond & Clarke, 1988). Hence, it is likely that BRL37344 is producing a non-specific stimulation, and that β_3-adrenoceptors are not present on bovine cultured adrenal ZFR cells.

Secondly, determination of the pA_2 values for the β_1-antagonist practolol and the β_2-antagonist ICI118,551 provided definitive evidence for the presence of β_1-receptors (Table 1). The pA_2 value for practolol of 6.85 (6.67–7.06) agrees well with published values (Table 1). The gradient of the Schild regression was significantly less than 1, slope = 0.90 (0.84–0.96), suggesting deviation from an ideal competitive antagonist. Practolol and ICI118,551 showed no partial agonist activity (results not shown), even though practolol is known to be a partial agonist in other systems (Kenakin & Black, 1978). It is possible that isoprenaline potentiated the partial agonist properties of practolol, leading to production of more hydrocortisone than expected and hence giving a Schild regression < 1.

The pA_2 value for the action of ICI118,551 at β_2-receptors has been found to be 9.26, and at β_1-receptors 7.17 (Bilski *et al.*, 1983). Hence, our experimental value of 7.14 (7.03–7.28) agrees well with the value for β_1-receptors. In this case, the gradient of the regression line of 0.99 (0.97–1.01) implies that ICI118,551 is acting as a pure competitive antagonist.

Although the occurrence of adrenergic control of steroidogenesis at the level of the adrenal cortex still remains to be established *in vivo*, an increasing number of observations suggest this possibility. Adrenergic innervation of the adrenal cortex has been demonstrated in man (Mikhail & Amin, 1969), the rat (Kleitman & Holzwarth, 1985) and the mouse (Migally, 1979). No similar studies on bovine adrenal cortex have been published.

Shima *et al.* (1984) have shown, using binding of [^{3}H]-dihydro-alprenolol, that membranes prepared from both the capsulated (zona glomerulosa, ZG) and decapsulated (ZFR) regions of the rat adrenal cortex contain β_1-adrenoceptors, and that only the latter are coupled to adenylate cyclase *in vitro*.

Previous work on primary cultures of adrenocortical cells has shown a steroidogenic response to catecholamines in rat ZG cells (DeLean *et al.*, 1984), in a bovine mixed adrenocortical preparation (Kawamura *et al.*, 1984), and in bovine purified ZFR cells (Walker *et al.*, 1988).

In conclusion, we have shown that β_1-adrenoceptors are responsible for mediating catecholamine-stimulated hydrocortisone secretion from primary cultures of bovine adrenal ZFR cells. Ungar (1979) suggested that β_1-adrenoceptors tend to be associated with adrenergic nerve endings so that the occurrence *in vivo* of catecholamine-stimulated steroidogenesis is probably mediated by direct adrenergic innervation of adrenocortical cells.

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Acetylcholine stimulates cortisol secretion through the M3 muscarinic receptor linked to a polyphosphoinositide-specific phospholipase C in bovine adrenal fasciculata/reticularis cells

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Summary

Zona fasciculata/reticularis (ZFR) cells, isolated from the bovine adrenal cortex, secreted cortisol in response to acetylcholine (AcCh). The response was present in freshly isolated cells and in cells maintained in primary culture, reaching a maximum after 48–72 h and thereafter declining. Cells maintained in primary culture for 72 h secreted cortisol with an ED_{50} at 1.2×10^{-6} M.

The potent inhibition of AcCh-stimulated secretion by atropine, and the relative ineffectiveness of nicotine or nicotinic antagonists, were consistent with a predominantly muscarinic response to AcCh in these cells. A selective M1-receptor agonist, McN-A-343, had no effect on cortisol secretion whereas the M3 antagonist, hexahydro-sila-difenidol, produced a dose-dependent inhibition of AcCh-stimulated cortisol secretion. These findings are consistent with AcCh mediating its effects on cortisol secretion through an M3 receptor.

While AcCh had no effect on cAMP formation, a dose-dependent increase in [^{3}H]phosphoinositols (identified using high-performance liquid chromatography (HPLC)) occurred in a manner that was not dependent on an influx of extracellular Ca^{2+}. Detailed HPLC analysis of the formation of ^{3}H-labelled phosphoinositols and glycerophosphoinositols from pre-labelled cells over the period 0–15 min showed that the earliest significant rise was in Ins(1,4,5)P_3 at 5 s, followed by later rises in $InsP_1$, $InsP_2$ and Ins(1,3,4)P_3. Additional studies using cells loaded with fura-2 indicator revealed a 1.6-fold increase in $[Ca^{2+}]_i$ from a mean resting value of 75 nM in response to 10^{-4} M AcCh. Furthermore, the rise in Ca^{2+} was not abolished by lowering extracellular Ca^{2+} to resting cytosolic levels, suggesting the mobilisation of an intracellular pool. These observations indicate that AcCh promotes rapid activation of a Ca^{2+}-independent and polyphosphoinositide-specific phospholipase C, and that the Ins(1,4,5)P_3 formed releases Ca^{2+} from an intracellular pool. The stimulation by AcCh of this signal transduction mechanism is consistent with our conclusion, based on the effects of the selective muscarinic agonist and antagonist on cortisol secretion, that the AcCh receptor is of the M3 subtype.

We conclude that AcCh, acting through an M3 receptor coupled to phospholipase C, regulates cortisol secretion at the cellular level in bovine adrenal ZFR cells.

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Introduction

There is an extensive literature on the ability of acetylcholine (AcCh) to stimulate catecholamine secretion from adrenomedullary cells, whereas there is little available information on the cholinergic response of adrenocortical cells. Rosenfeld (1955) provided the first evidence for muscarinic cholinergic stimulation of cortisol secretion in the isolated perfused calf adrenal gland. Although a nicotinic response was later described in cat adrenal cortex, the effects of AcCh itself or other cholinergic agonists and antagonists were not tested (Rubin and Warner, 1975), so the presence of a muscarinic receptor could not be excluded. Muscarinic, cholinergic regulation of adrenocortical steroidogenesis is not confined to mammals, but has also been observed, using a perifusion technique, in frog adrenal (inter-renal) tissue (Benjamina et al., 1987).

Crude membrane preparations of bovine adrenocortical tissue have been shown to contain binding sites for the muscarinic antagonist, L-quinuclidinyl benzylate (K_d of 1.2 nM), while trypsin-dispersed cells derived from bovine zona fasciculata/reticularis (ZFR) have been shown to secrete cortisol in response to AcCh via a muscarinic receptor (Hadjian et al., 1981, 1982). A muscarinic steroid response has also been reported in both cultured bovine adrenocortical cells (whole cortex) (Kawamura et al., 1985) and in freshly dispersed bovine zona glomerulosa (ZG) cells (Kojima et al., 1986).

The second messenger response to cholinergic stimulation of adrenocortical cells has been investigated by Hadjian et al. (1984) and by Kojima et al. (1986). Using bovine ZFR cell suspensions, Hadjian et al. (1984) showed that AcCh induced a dose-dependent increase in ^{32}P-labelling of phosphatidylinositol, first detectable at 2 min and abolished in the presence of muscarinic antagonists but not nicotinic antagonists. The rapid disappearance of prelabelled phosphoinosites was not investigated, however, and definitive evidence of activation of a phospholipase C was not provided. Using bovine ZG cells prelabelled with [^{3}H]inositol, Kojima et al. (1986) demonstrated that carbachol induced a rapid increase in formation of [^{3}H]inositol trisphosphate (isomer(s) not characterised), with slower increases in formation of labelled inositol bis- and monophosphate species, a response characteristic of the activation of a polyphosphoinositide-specific phospholipase C. Both Hadjian et al. (1982) and Kojima et al. (1986) found that AcCh and carbachol, respectively, failed to elicit any increase in adenosine 3,5-cyclic monophosphate (cAMp) production at maximal steroidogenic concentrations of either agonist. The former group also reported no change in guanosine 3,5-cyclic monophosphate above basal levels.

It has recently become clear that the pharmacological subdivision of the muscarinic receptor into M1, M2 (cardiac) and M3 (glandular) subtypes (Levine and Birdsall, 1989) is reflected in different subtypes of muscarinic AcCh receptor mRNA species (mAChR). Indeed, four unique mAChR subtypes have been identified, designated mAChR I–IV. The receptor subtypes corresponding to mAChR I and mAChR III appear to act through phosphoinositide hydrolysis and to equate with the M1 and M3 subtypes, as defined pharmacologically (Fukuda et al., 1988). The receptor subtype involved in the muscarinic stimulation of adrenocortical steroidogenesis is not known.

In studying the effects of AcCh on ZFR function, it is important to use a preparation free from ZG cell contamination; AcCh is known to stimulate aldosterone secretion from bovine ZG cells (Kojima et al., 1986) and other released steroids (e.g. progesterone) could potentially serve as steroidogenic precursors for cortisol production from the ZFR cells. The extent of glomerulosa cell contamination has not been investigated in the several reports of the effects of AcCh on cortisol production from bovine ZFR cells.

By employing the column purification method described by McDougall et al. (1979), we have previously shown that it is possible to obtain ZFR cell preparations which have undetectable glomerulosa cell contamination (Williams et al., 1989). Using this highly responsive culture system for purified bovine ZFR cells (Williams et al., 1989) we now show that the cortisol response to AcCh is mediated by the M3 receptor. High-performance liquid chromatography (HPLC) analysis of the water-soluble products of phosphoinositide hydrolysis confirmed that the M3 receptor is cou-

pled to a polyphosphoinositide-specific phospholipase C. Studies on the Ca^{2+} sensitivity of this response and the direct effect of AcCh on intracellular [Ca^{2+}] are also reported.

Materials and methods

The preparation of bovine adrenocortical ZFR cells, and the radioimmunoassay of cortisol and cAMP have been described elsewhere (Williams et al., 1989). The materials used for the measurement of ^{3}H-labelled phosphoinositols and glycerophosphoinositols were as described by Bird et al., 1989a), and for the small-scale periodate oxidation of the putative glycerophosphoinositol peaks from the detailed HPLC time-course are also described (Bird et al., 1989b). The partisphere SAX-5 columns were obtained from Jones Chromatography. [*myo*-^{3}H]Inositol and the [*myo*-^{3}H]phosphoinositol standards were obtained from Amersham International, Aylesbury, U.K. Hydroluma scintillation fluid was from May and Baker, Eccles, U.K. The fura-2 acetoxymethyl ester (AM) was obtained from BCL, Lewes, U.K. The AcCh, carbachol, pirenzipine, nicotine, atropine, tubocurarine and the CPSR5 (controlled serum replacement) were from the Sigma Chemical Co., Poole, U.K. Angiotensin II (Asp^1-Val^5) (AII) was the WHO International Standard from the National Institute for Biological Standards and Control. The M1 agonist McN-A-343 and the M3 antagonist, hexahydro-sila-difenidol hydrochloride, were obtained from Drs. G. Lambrecht, E. Mutschler and R. Tacke of the Johann-Wolfgang-Goethe University, Frankfurt am Main, F.R.G.

All other chemicals were of Analar grade and obtained from BDH, Poole, U.K. and the Aldrich Chemical Co., Gillingham, U.K.

Preparation, purification and culture of bovine adrenocortical ZFR cells

Bovine adrenal glands were obtained from freshly slaughtered 1 to 2 year-old steers at the local abattoir. The details of the tissue digestion (ZFR slices), the column purification step and the properties of the cell preparation are described by Williams et al. (1989). Purified cells were plated out at 250–300,000 cells/ml in 12-well plates in Ham's F10 medium supplemented with 10% fetal calf serum (or controlled replacement serum), penicillin (50 IU/ml), streptomycin (50 μg/ml) and amphotericin B (2.5 μg/ml). Addition of [^{3}H]inositol label (10 μCi/ml) is most conveniently carried out at 24 h when the growth medium is changed. Previous work with these cells had established that steady-state labelling was reached 42 h after label addition. Therefore the second messenger and steroid studies have been regularly carried out at 72 h (Bird et al., 1989a).

Agonists and antagonists were made up and diluted in Earle's balanced salt solution (EBS) containing 0.1% added glucose and 0.2% added bovine serum albumin (BSA) (EBS/BSA/glucose). In the case of agonists and antagonists used for the studies of phosphoinositide hydrolysis, LiCl (10 mM) and inositol (10 mM) were also present (note that neither LiCl nor inositol were present when measuring the effects of the different agonists/antagonists on cortisol secretion). The solutions were added in 50 μl to each well to achieve a final volume of 500 μl. In experiments with both antagonists and agonists, the antagonist was added 5 min before the appropriate agonist. Experiments investigating the effects of different agonists/antagonists on cortisol production, cAMP production and ^{3}H-labelled total head groups were carried out on at least three occasions, unless otherwise stated.

Cortisol and cAMp production

The procedure for measuring the cortisol output from the cultured cells and the details of the cortisol assay are described in Walker et al. (1989).

In some experiments cAMP levels in both medium and cells were measured at 15 min as described by Williams et al. (1989). For these experiments, the dose range of AcCh used to investigate the cAMP response was the same as the dose range used to investigate the cortisol response (i.e. 10^{-7} to 10^{-3} M AcCh). In these experiments, ACTH at 10^{-10} M served as a positive control stimulus.

Measurement of phospholipase activation (measured as total [^{3}H]head groups formation)

The cell labelling with [^{3}H]inositol, the execution of the agonist/antagonist experiments at 72 h

in the presence of Li^+ and the subsequent recovery and measurement of water-soluble ^3H-labelled total head groups (i.e. total ^3H-labelled phosphoinositols and glycerophosphoinositols) have been described by Bird et al. (1989a).

HPLC analysis of ^3H-labelled phosphoinositols and glycerophosphoinositols

To the neutralised aqueous samples (0.9 ml each) containing the labelled products (processed according to Bird et al., 1989a), 1 mg mannitol was added to act as carrier and the samples lyophilised. Each dried sample was then dissolved in 1.5 ml EDTA (1 mM) and stored at −20°C prior to analysis.

HPLC analysis was carried out using a 25 cm partisphere SAX-5 column with a 1 cm SAX-5 guard column pre-equilibrated with H_2O alone. Each sample was made up to 2 ml with 0.5 ml of a mixture of ATP/ADP/AMP (each at 0.1 mg/ml) in 1 mM EDTA. The flow-rate was kept at 1 ml/min throughout. At 5 min after sample injection, gradient elution with 1 M ammonium formate (pH 3.7 using orthophosphoric acid) (solution B) was started, rising to 100% B at 35 min and remaining at 100% for the next 5 min. Fractions (0.5 ml) were collected throughout. ^3H counts in each fraction were measured after addition of 3 ml scintillation fluid, counting for 2 min in a Packard 1900 scintillation counter with 20 cpm background correction and correcting for chemiluminescence.

The identical procedure was also carried out with radiolabelled Ins1P, Ins(1,4)P_2 and Ins(1,4,5)P_3 standards in order to assist peak identification. Where AcCh-stimulated extracts were processed to destroy glycerophosphoinositols with sodium periodate, unlabelled GroPInsP$_n$ mix was added to 0.5 mM as cold carrier and the procedure described by Bird et al. (1989b) followed. Prior to HPLC analysis of the periodate-oxidized samples, dimethylhydrazine derivatives were first removed by passing the sample through a C-18 reverse-phase SepPak cartridge.

Measurement of water-soluble [^3H]head groups in low and normal Ca^{2+}-containing medium

To investigate the Ca^{2+}-dependency of the production of ^3H-labelled total aqueous head groups, experiments were carried out as above in medium with normal extracellular Ca^{2+} (1.8 mM, present in EBS) and modified medium for which the free Ca^{2+} concentration had been adjusted to 120 nM according to the EGTA/Ca^{2+} buffer system described by Renard et al. (1987). Pre-labelled cells were stimulated for 5 min with 10^{-4} M AcCh in medium of normal or low Ca^{2+} content. The reaction was stopped and the perchloric acid extracts processed as previously described (Bird et al., 1989a).

Measurement of intracellular [Ca^{2+}]

Cells were prepared as described above, but kept in suspension culture, rather than plating out, at a concentration of 10^6 cells/ml in Ham's F10 (containing 10% CPSR5 serum replacement) over 72 h. The cells in suspension culture could be shown to be viable at 72 h on the basis of their responsiveness to a range of agonists which included ACTH, All and AcCh.

At 72 h, the cells were harvested, centrifuged at $450 \times g$, washed in modified Krebs-Ringer buffer containing 0.5% BSA and 0.1% added glucose (Capponi et al., 1986), and resuspended in the same buffer at 5×10^6 cells/ml. Cells were loaded at this density with 15 μM fura-2 AM for 30 min at 37°C. 1 ml portions of the fura-2 loaded cells were then centrifuged at $450 \times g$ for 2 min in Eppendorf tubes and the pellet resuspended in 2 ml of modified Krebs-Ringer buffer (without BSA) to give a final cell density of 2.5×10^6 cells/ml, using 2 ml per cuvette. Fluorescence measurements were carried out at an excitation wavelength of 340 nm, recording the fluorescence signal at the emission wavelength of 505 nm on a Perkin-Elmer model LS-5 fluorimeter with continuous stirring at 37°C. Agonists and antagonists were added in a volume of 20 μl to give the appropriate final concentrations. Details of the calibration for the determination of intracellular Ca^{2+} are as described by Capponi et al. (1986), but substituting digitonin for Triton-X.

Statistics

Tests of statistical significance employed Student's *t*-test.

Results

Day-by-day cholinergic response of cultured bovine adrenal ZFR cells

Preliminary experiments established that maximal cortisol secretion of the bovine ZFR cells was achieved by 10^{-4} M AcCh. Accordingly, this dose of AcCh was used to measure cortisol secretion (over 60 min) in the freshly isolated cells (0 h) and at 24, 48, 72 and 96 h in culture. The pre-culture (0 h) experiments were all carried out in EBS/BSA/glucose before cells had been exposed to any growth medium. Fig. 1 shows the day-to-day changes in both basal and AcCh-stimulated cortisol secretion for cells grown in Ham's F10 supplemented with 10% fetal bovine serum, 10% heat-treated (57°C for 45 min) fetal bovine serum (same batch) or 10% CPSR5 serum replacement (Sigma).

Responsiveness was found to be greatest in CPSR5 serum substitute, and better in the heat-treated serum as compared to unheated serum. All subsequent studies of the cholinergic response were undertaken in Ham's F10 medium containing 10% (v/v) CPSR5. Although maximal responsiveness occurred at 48 h, all experiments have been carried out at 72 h to conform to the relatively long period (42 h) necessary to achieve steady-state labelling of these cells with [*myo*-^{3}H]inositol (Bird et al., 1989a). Label was added at 24 h in culture at the time of the medium change.

Characterization of the cholinergic receptor

Fig. 2 shows the dose-dependent secretion of cortisol in response to AcCh, nicotine and McN-A-343. No response was observed to the M1 agonist, McN-A-343. Although nicotine did elicit some cortisol secretion in the experiment illustrated, this was not achieved until 10^{-5} M and even then the maximum was $< 25\%$ of that to AcCh. The threshold response to AcCh was between 10^{-7} M and 10^{-6} M (in four experiments) rising steeply to reach a maximum by 10^{-4} M. The ED_{50} for AcCh-stimulated cortisol secretion at 72 h was 1.2×10^{-6} M (range: 3.7×10^{-7} M to 3.7×10^{-6} M; $n = 4$ experiments). Stimulation of cortisol secretion was also observed in two experiments with the synthetic muscarinic agonist, bethanechol (data not shown).

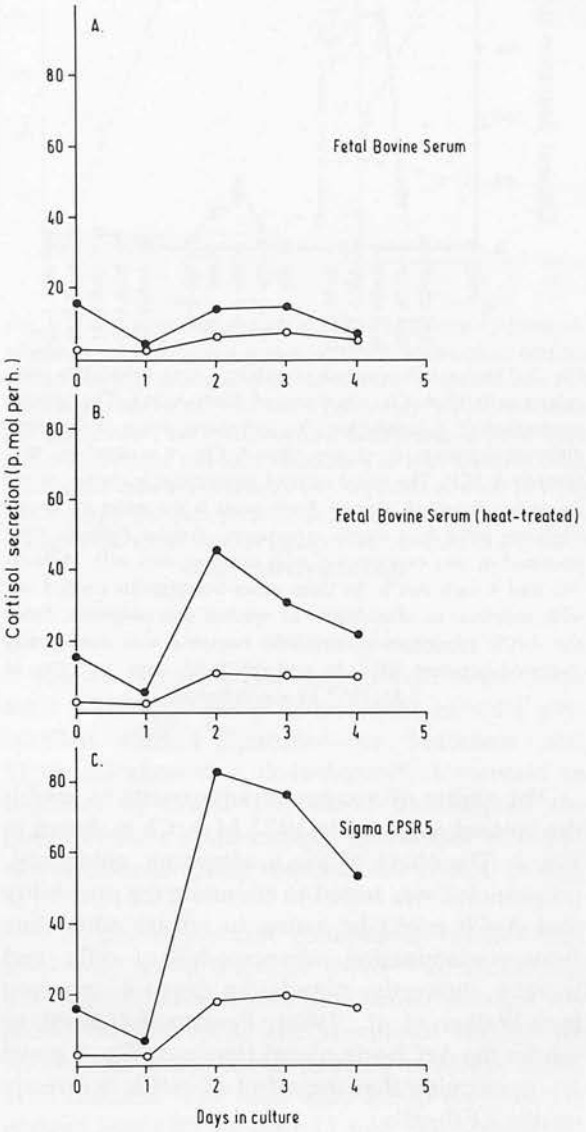

Fig. 1. Dependence of the day-by-day cortisol response to AcCh on growth conditions. Cells were isolated and plated out as described in Materials and Methods. For the freshly isolated cells (in suspension) and at 24, 48, 72 and 96 h later (in culture), the basal cortisol response (○) and the response to 10^{-4} M AcCh (●) were measured over a 1 h incubation. The graphs illustrate the responsiveness of cells grown in the presence of 10% fetal bovine serum (*A*), 10% heat-treated fetal bovine serum (*B*) and 10% serum replacement CPSR5 (Sigma) (*C*), all using Ham's F10 as growth medium. Each point is the mean of a triplicate determination. A very similar picture was also found in a second experiment in which 10^{-4} M carbachol substituted for AcCh.

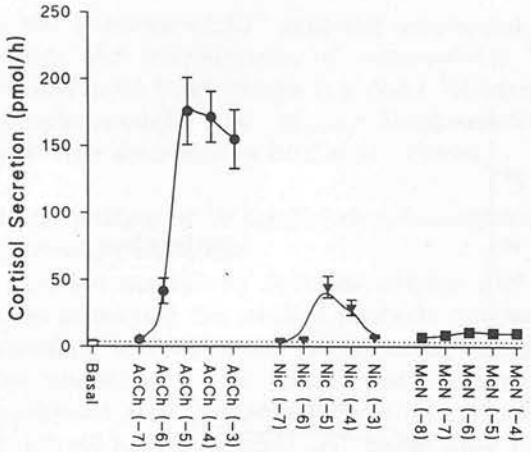

Fig. 2. The cortisol response of cultured zona fasciculata/reticularis cells to AcCh, nicotine and McN-A-343. The cortisol production (1 h incubation) for increasing doses of the three different agonists is shown (● = AcCh; ▼ = nicotine; ■ = McNeil-A-343). The basal cortisol production is shown as the single bar (labelled 'Basal'). Each point is the mean ± S.D. for triplicate wells in a single experiment. Similar findings were obtained in two experiments with nicotine, two with McN-A-343 and 4 with AcCh. In three other experiments carried out with nicotine, no stimulation of cortisol was observed. From the AcCh experiments, threshold response was consistently observed between 10^{-7} M and 10^{-6} M, with an ED_{50} of 1.2×10^{-6} M acetylcholine.

The ability of a range of antagonists to inhibit the cortisol response to 10^{-4} M AcCh is shown in Fig. 3. The effect of the β-adrenergic antagonist, propranolol, was tested to eliminate the possibility that AcCh might be acting to release adrenaline from contaminating adrenomedullary cells, and thereby indirectly stimulating cortisol secretion (see Walker et al., 1988). Propranolol failed to inhibit the AcCh-stimulated response (Fig. 3, panel *A*), confirming that the effect of AcCh is directly on the ZFR cells.

The AcCh response is dose-dependently inhibited by the muscarinic antagonist atropine (Fig. 3, panel *A*). Significant inhibition of the response is evident at 10^{-8} M atropine and is $> 60\%$ by 10^{-7} M atropine, at which concentration the nicotinic antagonist, tubocurarine, has no effect on AcCh-stimulated cortisol production. Indeed, inhibition by tubocurarine is still minimal at 10^{-4} M, while an equal concentration of atropine inhibits stimulated cortisol secretion to basal levels. These results are therefore consistent with the presence of a predominantly muscarinic, cholinergic receptor in these cells.

Pirenzipine, an M1 antagonist, produced inhibition of the AcCh-stimulated cortisol response, but only at concentrations of 10^{-5} M pirenzipine or greater (results not shown). Furthermore, this

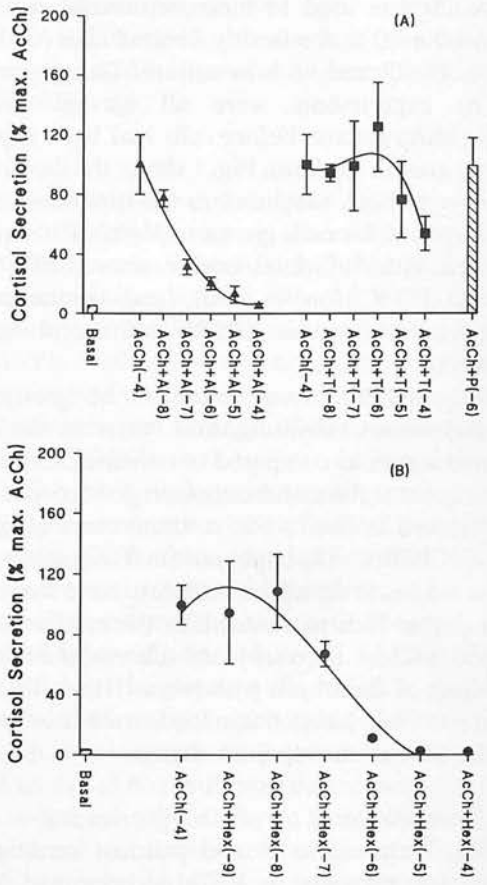

Fig. 3. The effects of different antagonists on AcCh-stimulated cortisol secretion. The cortisol response (1 h incubation) was measured for each agonist, antagonist or combination, expressing the secretion as a percentage of the response achieved by 10^{-4} M AcCh (100%). Panel *A* shows the effect of the muscarinic antagonist, atropine (A) (▲) and the nicotinic antagonist, tubocurarine (T) (■). The basal response alone is shown as the single bar ('Basal'). The effect of propranolol (P) (at 10^{-6} M) on the AcCh-stimulated response is shown in the last bar of the figure. Similar findings were obtained in three such separate experiments. Panel *B* shows the effect of the M3 antagonist, hexahydrosila-difenidol (Hex) (●). The basal response alone is shown as the single bar ('Basal'). All values are the mean ± SD for triplicate wells in a single experiment. Similar results were obtained in a total of two such experiments. In all cases, no effect of the antagonist alone on basal cortisol secretion was found.

antagonist was much less effective, even when compared with concentrations of atropine 10-fold lower at 10^{-6} M. Previous studies have shown that the antimuscarinic potencies of pirenzipine and atropine at M1 receptors are very similar, whereas atropine is almost 100-fold more potent at M2 or M3 receptors (Lambrecht et al., 1985). Hence, these findings argue strongly that the cholinergic response of the ZFR cells is mediated via an M2 or M3 receptor.

Fig. 3 (panel *B*) shows that the M3 receptor antagonist, hexahydro-sila-difenidol, produced a dose-dependent decrease in cortisol response consistent with AcCh acting through the M3 receptor.

Second messenger responses to AcCh

No change in cAMP production was observed in response to the full dose range of AcCh used to study cortisol secretion. Under these conditions, the positive control, $ACTH_{1-24}$, elicited a clear cAMP response (results not shown).

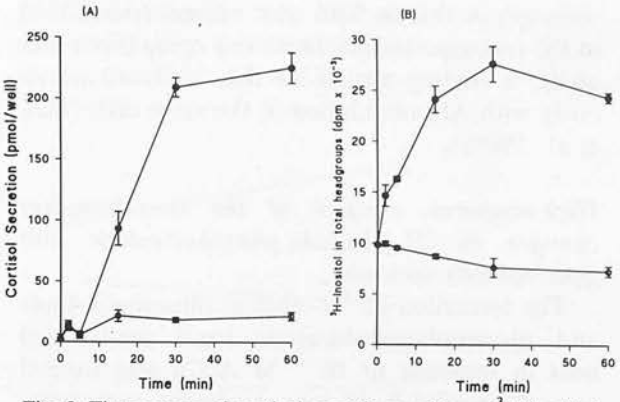

Fig. 5. Time-course of cortisol secretion and [*myo*-^{3}H]inositol-labelled total head group response. The time-course of cortisol production was measured (72 h in culture) in the absence (○) or presence (●) of 10^{-4} M AcCh (graph *A*). Within the same cell preparation, the corresponding time-course of total head group production was also measured (72 h in culture) using pre-labelled cells in the absence (○) or presence (●) of 10^{-4} M AcCh (graph *B*). Each point is the mean ± SD of three wells in a single experiment. Similar results were obtained in a total of three such experiments.

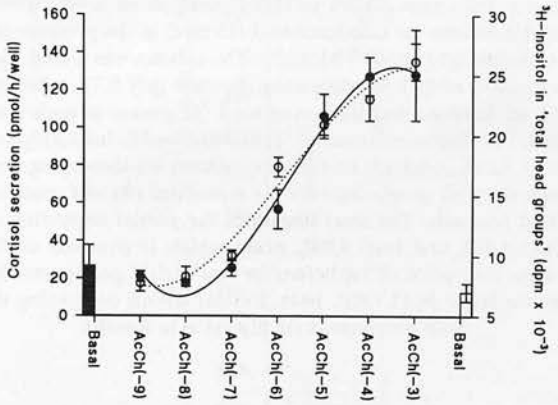

Fig. 4. Comparison of AcCh-stimulated cortisol secretion and [*myo*-^{3}H]inositol-labelled total head group response. At 72 h in culture, unlabelled cells were stimulated with increasing concentrations of AcCh and the cortisol output measured (1 h incubation), as described (solid line; ●). The basal cortisol output over this period is also shown as the shaded bar ('Basal'). [*myo*-^{3}H]Inositol-labelled cells were stimulated with the same concentration range of AcCh for 15 min and the incorporation of label into total water-soluble head groups measured (broken line; ○). The basal total head group response is shown as the unshaded bar ('Basal'). Each data point is the mean ± SD of three wells for a single cell preparation in which the cortisol and total head group responses could be compared. Similar results were obtained in three such experiments.

Phospholipase activation (reflected as increased total [^{3}H]-head group production in cells pre-labelled with [^{3}H]inositol–see Materials and Methods) showed a dose-dependent increase in response to AcCh. The close similarity of the dose–response relationships for cortisol secretion and total head group response are evident in Fig. 4.

The time-dependent changes in cortisol and total head group response to 10^{-4} M AcCh and the corresponding basal (no AcCh) changes are illustrated in Fig. 5. The stimulated cortisol response was linear for at least 30 min, but thereafter declined. The total head group response remained linear for at least 15 min and also subsequently declined. The response was statistically significant ($P < 0.005$) when compared to the basal response at the earliest time-point measured (2 min); the stimulated cortisol secretion only reached statistical significance by 15 min.

The total head group response was shown to occur in the absence of Ca^{2+} influx in three experiments carried out in EBS containing free [Ca^{2+}] buffered to 120 nM ($P < 0.05$; $n = 3$) (Renard et al., 1987). The total head group response was, however, diminished to 19.25 ± 0.75% (mean

± range) of that in EBS with normal free [Ca^{2+}] in the two experiments where this comparison was made, a finding similar to that observed previously with AII stimulation in the same cells (Bird et al., 1989a).

High-resolution analysis of the time-dependent changes in ^{3}H-labelled phosphoinositols and glycerophosphoinositols

The formation of ^{3}H-labelled phosphoinositols and glycerophosphoinositols from pre-labelled cells in response to 10^{-4} M AcCh was studied over the period 0–15 min, analyzing each time-point by HPLC (Fig. 6). Peak identification was assisted by determining the elution positions of the three radiolabelled standards Ins1P, Ins(1,4)P_2 and Ins(1,4,5)P_3. The three peaks eluting after the Ins(1,4,5)P_3 standard were assigned to $InsP_4$, $InsP_5$ and $InsP_6$ (Fig. 6). Provisional assignment of the peaks eluting immediately before the Ins1P and Ins(1,4)P_2 standards as GroPIns and GroPIns4P, respectively, was subsequently confirmed by periodate oxidation. This process selectively removes the glycerol backbone of such compounds, converting them to their corresponding phosphoinositols. After periodate oxidation of a stimulated sample (15 min) and subsequent HPLC analysis, the putative GroPIns and GroPIns4P peaks had disappeared, the products presumably now migrating with the InsP and $InsP_2$ peaks.

Fig. 6 shows that the cells stimulated by 10^{-4} M AcCh show much increased formation of [^{3}H]inositol mono-, bis- and trisphosphates, but not of the glycerophosphoinositols. Thus AcCh must stimulate the activation of a phospholipase C and not, for example, a phospholipase A_2.

On the partisphere SAX-5 column, it was not possible to resolve the InsP isomers into Ins1P or Ins4P; neither could the $InsP_2$ peak be resolved into isomers. Accordingly, these peaks are simply labelled InsP and $InsP_2$ in Fig. 6. However, the same column was able to resolve $InsP_3$ into two peaks, one co-eluting with the Ins(1,4,5)P_3 standard and assigned, therefore, this isomer status and the other eluting slightly ahead of the standard and presumed to be Ins(1,3,4)P_3. Further support for the identity of this peak as Ins(1,3,4)P_3 rather than as GroPIns(4,5)P_2, which has a retention time between that of Ins(1,4)P_2 and

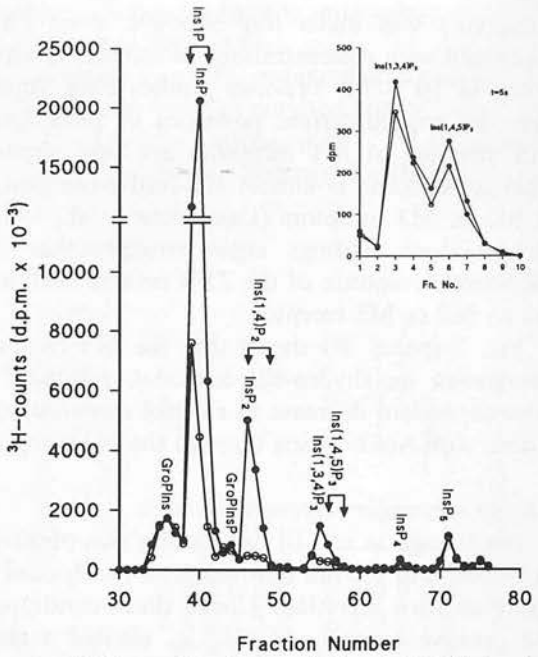

Fig. 6. HPLC profile of [*myo*-^{3}H]inositol-labelled phosphoinositols and glycerophosphoinositols at 15 min stimulation with 10^{-4} M AcCh. The water-soluble extracts containing [*myo*-^{3}H]inositol-labelled phosphoinositols and glycerophosphoinositols were subject to HPLC analysis on a partisphere SAX-5 column for cells incubated (15 min) in the presence (●) or absence (○) of 10^{-4} M AcCh. The column was eluted with a gradient of 0–1 M ammonium formate (pH 3.7), collecting 0.5 ml fractions and measuring total ^{3}H counts in each fraction. The elution positions of ^{3}H-labelled Ins1P, Ins(1,4)P_2 and Ins(1,4,5)P_3 standards on the same column are shown, together with the peak assignments for the stimulated (●) and unstimulated (○) cells. The inset illustrates the partial separation of Ins(1,3,4)P_3 and Ins(1,4,5)P_3 peaks which is observed at the earlier time-point of 5 s, before the Ins(1,4,5)P_3 peak is masked by the larger Ins(1,3,4)P_3 peak. Further details concerning the peak assignments are discussed in Results.

Ins(1,4,5)P_3, is provided by its observed resistance to periodate oxidation.

At the 15 min time-point, the two $InsP_3$ peaks were not clearly resolved; most of the $InsP_3$ peak in the stimulated sample is made up of Ins(1,3,4)P_3 at this time and masks the much smaller radiolabelled Ins(1,4,5)P_3 peak. At the earliest time-points, when the Ins(1,3,4)P_3 peak was not so large, it was possible to resolve the two isomers (see Fig. 6, inset) and monitor increased formation of Ins(1,4,5)P_3 and Ins(1,3,4)P_3 separately in the stimulated samples (Fig. 7).

Fig. 7 shows the full time-course for both the unstimulated and stimulated samples, measuring the radioactivity in the peaks for InsP, $InsP_2$, $Ins(1,3,4)P_3$/$Ins(1,4,5)P_3$, $InsP_4$ and $InsP_5$ for each time-point. The earliest significant change in response to 10^{-4} M AcCh was a rise in [^{3}H]-

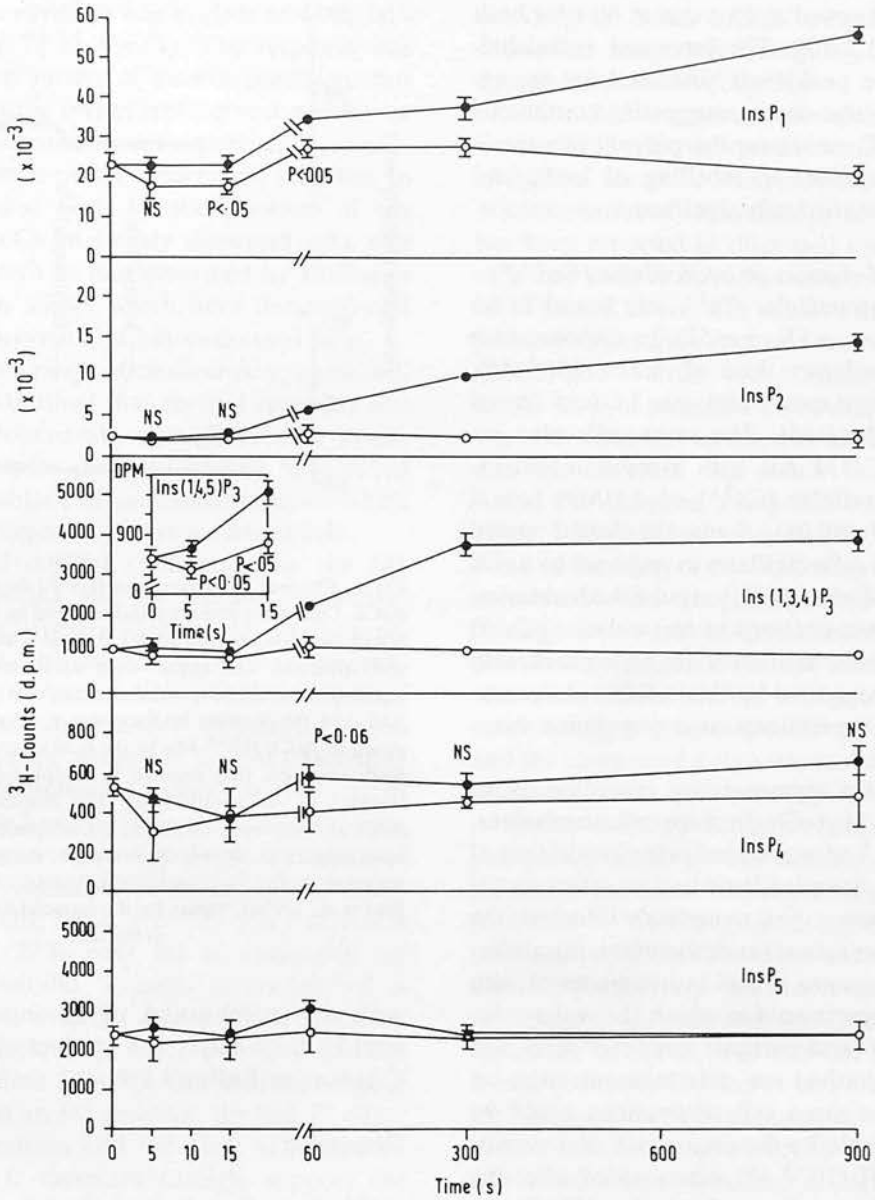

Fig. 7. Time-course of appearance of ^{3}H-labelled phosphoinositol species in response to AcCh. Pre-labelled cells were exposed (●) or not exposed (○) to AcCh (10^{-4} M) (72 h in culture), terminating each incubation over the time period 0 to 900 s. The water-soluble extracts at each time-point were subject to HPLC analysis on a partisphere SAX-5 column, and the ^{3}H counts in the peaks identified (using appropriate standards) as InsP, $InsP_2$, $Ins(1,4,5)P_3$, $Ins(1,3,4)P_3$, $InsP_4$ and $InsP_5$,separately measured. The figure shows the time-course of appearance of ^{3}H counts in these different phosphoinositol species. Each point is the mean ± SD of three HPLC analyses from three separate wells. The earliest time-point at which the stimulated cells differ significantly from the unstimulated cells for each phosphoinositol species is shown. Note that the stimulated $InsP_2$ is significantly different from control at 60 s. In the case of $Ins(1,4,5)P_3$, stimulated cells achieve significant difference from control cells at 5 s and 15 s; this is shown in the inset graph with the expanded scale.

$Ins(1,4,5)P_3$ at 5 s, consistent with a phospholipase C-dependent hydrolysis of $PtdIns(4,5)P_2$ as the earliest event in response to AcCh stimulation. Significant increase in radiolabelling of InsP was subsequently observed at 15 s and at 60 s for both $InsP_2$ and $Ins(1,3,4)P_3$. The increased radiolabelling in all these peaks was sustained for the remainder of the time-course, suggesting continuous phospholipase C action on the polyphosphoinositides. Small increases in labelling of $InsP_4$ and $InsP_5$ were not statistically significant.

Measurement of changes in intracellular $[Ca^{2+}]$

The basal intracellular Ca^{2+} was found to be 75 ± 3 nM (mean $\pm$SE, $n = 52$). In response to a maximally stimulatory dose of AcCh (10^{-4} M), the mean increase over basal was 1.6-fold ($n = 4$ experiments; $P < 0.05$). The same cells also responded to 10^{-8} M AII with a mean n-fold increase in intracellular $[Ca^{2+}]$ of 1.8-fold ($n = 4$ experiments; $P < 0.05$). Since the fura-2 signal from individual cells oscillates in response to AcCh (10^{-4} M) (Williams, B.C., unpublished observations), peak concentrations of intracellular $[Ca^{2+}]$ in individual cells are likely to be considerably higher than is suggested by this n-fold value, measured in these experiments as a population average.

Fig. 8 shows a representative recording in response to 10^{-4} M AcCh. In three cell suspensions, atropine at 10^{-6} M was added prior to addition of 10^{-4} M AcCh. Atropine itself had no effect on the fura-2 fluorescence, yet completely blocked the increase in fluorescence (and, therefore, intracellular Ca^{2+}) in response to AcCh, in agreement with the previous experiments in which the water-soluble head group (and cortisol) responses were also completely abolished at this concentration of antagonist. The same cell suspensions could be shown to be viable by the occurrence of a normal response to AII (10^{-8} M) when added after the AcCh, but in the presence of atropine (Fig. 8).

The fura-2 fluorescence in response to 10^{-4} M AcCh was also measured in Krebs-Ringer buffer in which the Ca^{2+} concentration was buffered to 120 nM (Renard et al., 1987). As with the total head group response, the increase in fura-2 fluorescence and, by inference, $[Ca^{2+}]_i$ was still present at this Ca^{2+} concentration. This is consistent

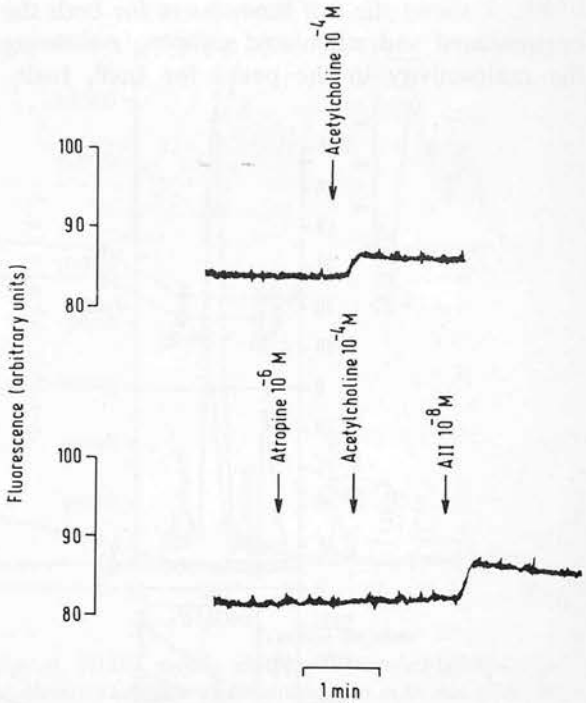

Fig. 8. Changes in intracellular $[Ca^{2+}]$ in cells stimulated by AcCh. Cells were prepared and cultured in suspension over 72 h and then loaded with fura-2 AM, as described in Materials and Methods. The upper trace illustrates the fluorescence signal from a cell suspension successfully loaded with fura-2 AM, and the increase in fluorescence when the cells are exposed to AcCh (10^{-4} M). In the bottom trace, a different cell suspension was first exposed to atropine (10^{-6} M) prior to addition of AcCh (10^{-4} M). The fluorescence response to AcCh is completely abolished by atropine, yet the same cell suspension was shown to be viable through its subsequent response to the Ca^{2+}-mobilising agonist, AII (10^{-8} M) (see Bird et al., 1989a). Values for the intracellular $[Ca^{2+}]$ are given in the text.

with the mobilization of an intracellular Ca^{2+} pool by $Ins(1,4,5)P_3$, the product of phospholipase C action on $PtdIns(4,5)P_2$.

Discussion

We have found a consistent steroid (cortisol) response to AcCh in our cultured bovine ZFR cells. This agrees with results of previous studies on purified bovine fasciculata/reticularis cells (Hadjian et al., 1982), unpurified adrenocortical cells from cat and bovine species (Rubin et al., 1975); Kawamura et al., 1985) and the perfused whole adrenal from calf (Rosenfeld, 1955).

The cortisol response was present in the freshly dispersed cells and was retained for at least 72 h in culture. At 24 h in culture, the cells were least responsive for any given concentration of AcCh. Maximal responsiveness was evident at 48 h, falling slightly by 72 h (Fig. 1). The response was present under a variety of growth conditions but was optimal in the serum replacement which was therefore used in subsequent experiments.

The occurrence of the cholinergic response in vivo is evidenced both by the presence of the response to AcCh in freshly dispersed cells and from studies, such as that described by Robinson et al. (1977) in sheep, which have demonstrated cholinergic innervation of adrenocortical cells.

Studies with a range of cholinergic agonists and antagonists established that cortisol secretion was occurring predominantly through binding to the muscarinic receptor. Previous studies have failed to clarify the subtype of muscarinic receptor which mediates this response in adrenocortical cells.

The lack of cortisol stimulation by the M1 agonist McN-A-343 and the relative ineffectiveness of the M1-specific antagonist, pirenzipine, were consistent with an M2 or M3 receptor subtype. M2 receptor stimulation would be expected to lead to a decrease in cAMP formation (Levine and Birdsall, 1989), while M1 and M3 receptors promote phosphoinositide hydrolysis (Fukuda et al., 1988). AcCh had no effect upon cAMP formation, in agreement with the findings of Hadjian et al. (1982) and Kojima et al. (1986), using bovine ZFR and ZG cells, respectively. In contrast, AcCh stimulation of ZFR cells led to catabolism of [^{3}H]phosphoinositides through activation of a phospholipase C with the dose-dependent formation of phosphoinositols which closely paralleled cortisol production (Fig. 4). The pharmacological evidence against an M1 receptor, the lack of effect on cAMP formation and the clear activation of phospholipase C therefore strongly support the involvement of the M3 receptor subtype in this response.

Further evidence for AcCh acting through the M3 receptor was obtained using the M3 receptor antagonist, hexahydro-sila-difenidol. Lambrecht and Mutschler (1985) presented evidence that hexahydro-sila-difenidol has high antimuscarinic potency at M3 receptors on the smooth muscle of ileum and urinary bladder, but low potency at myocardial M2 receptors. This antagonist produced a dose-dependent inhibition of the cortisol response to AcCh; there was little or no effect of the drug on basal cortisol output over the concentration range used, arguing against a non-specific toxic effect (Fig. 3).

While M3 receptors are clearly present on bovine ZFR cells, the question arises as to whether additional nicotinic receptors are also expressed. Nicotine-induced stimulation of steroidogenesis has been reported in dispersed cat adrenocortical cells but only at nicotine concentrations of 6×10^{-5} M and above. The relative magnitude of any possible muscarinic response was not investigated (Rubin and Warner, 1975). Although nicotine induced cortisol secretion in bovine ZFR cells, this was only observed in two out of five experiments, and the response was much less than that to AcCh. Furthermore, a response was only observed at 10^{-5} M nicotine or above (Fig. 2). Concentrations of nicotine $> 10^{-5}$ M were less potent and associated with morphological evidence of cell damage. L-(+)-tubocurarine also failed to antagonise AcCh-stimulated cortisol secretion, except at concentrations of 10^{-4} M or above. Together these results suggest a non-specific effect of nicotine, and the absence of a separate nicotinic, cholinergic receptor.

Earlier work on the involvement of membrane phosphoinositides in the cholinergic response of ZFR cells has been restricted to a measurement of increased ^{32}P labelling of phosphatidylinositol in response to AcCh. The data were insufficient to demonstrate clearly the involvement of phospholipase C in this response and the substrate specificity of the enzyme was not investigated. In bovine ZG cells, Kojima et al. (1986) investigated the time-course of appearance of [^{3}H]-phosphoinositols from pre-labelled bovine ZG cells and found a rapid (within 5 s) increased labelling of [^{3}H]inositol trisphosphate in response to carbachol. The use of a low-resolution chromatographic method by Kojima et al. (1986) precluded identification of inositol trisphosphate isomers and a study of the metabolic fate of the earliest formed inositol trisphosphate.

The earliest response to AcCh in ZFR cells is an increased labelling (within 5 s) of Ins(1,4,5)P_3

(Fig. 7) consistent with the earliest post-receptor event being a phospholipase C-dependent hydrolysis of PtdIns(4,5)P_2. At later time-points, Ins(1,3,4)P_3 formation increases (along with InsP_2 and InsP) and this isomer became the major contributor to total InsP_3 labelling. These observations confirm that PtdIns(4,5)P_2 catabolism is sustained throughout stimulation, and support the existence of the 'alternative' pathway in these cells, with activation of Ins(1,4,5)P_3 3-kinase and production of Ins(1,3,4,5)P_4 as an intermediate step.

The formation of Ins(1,4,5)P_3 results in many cells in the release of Ca^{2+} from an intracellular pool. In the studies using fura-2 loaded ZFR cells, basal $[Ca^{2+}]_i$ was 75 nM and increased 1.6-fold in the presence of 10^{-4} M AcCh. The same cells also responded to AII with an increase in $[Ca^{2+}]_i$ of 1.8-fold (Fig. 8). The response to AcCh, but not the response to AII was specifically blocked by 10^{-6} M atropine, confirming that the two agonists act through distinct receptors (Fig. 8). The initial rise in Ca^{2+} was shown to occur even in medium with $[Ca^{2+}]$ buffered to 120 nM (i.e., in the absence of Ca^{2+} influx) suggesting that mobilisation of an intracellular Ca^{2+} pool does indeed occur in these cells.

Cholinergic regulation of cortisol secretion has previously received little attention in vitro or in vivo. An unanswered question is the possible effect of AcCh on adrenocortical cell growth. Ashkenazi et al. (1989) reported that AcCh analogues stimulated DNA synthesis in astrocytes and transfected CHO cells expressing muscarinic receptors; the stimulation of DNA synthesis was specifically mediated via muscarinic receptor subtypes linked to phosphoinositide hydrolysis. The possible effects of AcCh on adrenocortical cell growth are currently under investigation.

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